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THE UNIVERSITY OF ALBERTA

MOLECULAR CLONING OF THE HUMAN LYMPHOCYTE DIFFERENTIATION ANTIGEN CD9

by

SHOU-CHING TANG

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

MEDICAL SCIENCES (MEDICINE)

EDMONTON, ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entiled MOLECULAR CLONING OF THE HUMAN LYMPHOCYTE DIFFERENTIATION ANTIGEN CD9 submitted by Shou-Ching Tang in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences.

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To my parents.

ABSTRACT

CD9 is a pre-B cell differentiation antigen which appears to be involved in the regulation of cell adhesion and signal transduction. The goal of this study was to clone the CD9 cDNA from a λgt11 cDNA expression library to further our understanding of its structure, and function.

CD9 antigen purified by monoclonal antibody affinity chromatography was used to raise a polyclonal antibody directed against the denatured protein. This antibody identified a putative cDNA clone encoding the CD9 protein from the hepatoma HepG2 λgt11 cDNA expression library. Rescreening of positive clones with polyclonal antibodies pre-adsorbed with cells expressing varying levels of CD9 antigen provided confirmatory evidence of specificity. A second clone was isolated from the pre-B cell NALM-6 λgt11 cDNA library using the first cDNA clone as hybridization probe. Sequencing of the entire insert on both strands was achieved by Sanger's dideoxy chain termination method using sequence-specific synthetic oligonucleotides as primers. The amino acid sequence predicted by the cDNA almost completely matched our peptide mapping data for the native CD9 protein, and did not contain a hydrophobic transmembrane domain consistent with CD9's status as a nonintegral membrane component. A search of the human gene data-base indicated that the cDNA is unique and related to the immunoglobulin supergene family. Southern-blots indicated that the gene may be a member of a multi-gene family, and showed a restriction fragment length polymorphism. The gene appears to be subjected to multiple levels of expression control since Northern and Western blots demonstrated that the mRNA is both transcribed and translated in cells which lack CD9 on their surface. Furthermore, Northern blots confirmed that CD9 expression is regulated in part by cell activation, since the mRNA is induced upon T-cell stimulation by mitogens, and by malignant transformation of NIH 3T3 cells by activated c-ras^{Ha} oncogene.

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ABBREVIATIONS AND DEFFINITIONS

A23187:	calcium ionophore
ALL:	acute lymphoblastic leukemia
AML:	acute myelogenous leukemia
ANLL:	acute non-lymphoblastic leukemia
BL:	Burkitt's lymphoma
BMT:	bone marrow transplantation
C-terminal:	protein carboxyl terminal
cALL:	common acute lymphoblastic leukemia
CALLA antigen:	common acute lymphoblastic leukemia antigen
cccDNA:	closed circular DNA
CD:	cluster of differentiation
cDNA:	complementary deoxyribonucleic acid
CLL:	chronic lymphoblastic leukemia
con A:	concanavalin A
DAG:	diacylglycerol
dd H_2O:	double-distilled water
Denhart's solution:	0.02% w/v each of Ficoll, polyvinylpyrollidone, and bovine serum albumin
DMEM medium:	high glucose Dulbeco's Modified Eagle Medium
DMSO:	dimethylsulfoxide
DNA:	deoxyribonucleic acid
dNTPs:	the four common deoxynucleotide triphosphate (G,A,T,C)
ds-cDNA:	double-stranded cDNA
DTT:	dithiothreitol
E. coli:	Escherichia coli

EDTA:	ethylenediaminetetraacetic acid
EGTA:	ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N",N'-tetraacetic acid
ELISA:	enzyme-linked immunosorbent assay
FACS:	fluorescence activated cell sorter
FCR:	fibrin clot retractile activity
FCS:	fetal calf serum
G protein:	GTP binding protein
GP Ib-IX:	platelet receptor for von Willebrand factor
GP IIb/IIIa:	heterodimer of glycoprotein IIb and IIIa, platelet receptor for fibrinogen
HLA antigen:	human leucocyte antigen
HPLC:	high performance liquid chromatography
HRP-GAM:	horse-radish peroxidase conjugated goat anti-mouse IgG antibody
HRP-GAR:	horse-radish peroxidase conjugated goat anti-rabbit IgG antibody
IgG:	immunoglobulin isotype G
IP_3:	inositol trisphosphate
IPTG:	isopropyl β-D-thiogalactopyranoside
kb:	kilo-base or 1,000 base pairs of double stranded DNA
kDa:	kilo-Daltons
KLH:	Keyhole Limpet Hemocyanin
Kozak sequence:	consensus nucleotide sequence surrounding the functional eukaryotic protein translation start codon ATG
λ diluent:	10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$
LB medium:	Lauri-Bertani bacterial culture medium
LDH:	lactic dehydrogenase
λgt11:	a cDNA expression vector derived from bacterial phage in which the translated foreign protein is fused to β-galactosidase

mAb:	monoclonal antibody
MHC antigen:	major histocompatibility antigen
MLR:	mixed lymphocyte reaction
MOPS:	20 mM 3-(N-morpholino) propane sulfonic acid pH 7.0
mRNA:	messenger ribonucleic acid
MW:	molecular weight
N-CAM:	neural cell adhesion molecule
NHL:	non-Hodgkin's lymphoma
NP-40:	Nonidet P-40
N-terminal:	protein amino terminal
OLB mix:	250 mM Tris-HCl pH 8.0, 25 mM MgCl2, 45 mM 2-ME, 1.0 M Hepes buffer pH 6.6, 100 μM each of dATP, dCTP dGTP, and dTTP, and 540 μg/ml of random primers
ORF:	open reading frame
Pansorbin:	10% suspension of heat killed and formalin fixed Staphylococcus aureus (Cowan I strain)
PBS:	phosphate buffered saline
PDGF:	platelet derived growth factor
PEG:	polyethylene glycol
pEXV-3:	a cDNA expression plasmid vector carrying SV40 promotor and ampicillin-resistant gene marker
p.f.u.	plaque forming unit
PGG_2:	prostaglandin G_2
PGH_2:	prostaglandin H_2
PHA:	phytohemagglutinin
PIP_2:	phosphatidylinositol bisphosphate
PKC:	protein kinase C
PLC:	phospholipase C

PMA:	phorbol-12-myristate-13-acetate, a phorbol diester
poly A^+ RNA:	cellular RNA enriched for mRNA by affinity chromatography over oligo-dT cellulose
PPD:	purified protein derivative of tuberculin
PRP:	platelet rich plasma
pSV2neo:	a cDNA expression plasmid vector carrying SV40 early promotor and neomycin-resistant gene marker
PWM:	pokeweed mitogen
QN^+:	hexadecyltrimethylammonium bromide
RGD sequence:	a tripeptide arg-gly-asp involed in cell adhesion
RNA:	ribonucleic acid
SAM:	S-adenosyl-L-methionine
SAS:	saturated ammonium sulfate
SDS:	sodium dodecyl sulphate
SDS-PAGE:	SDS-polyacrylamide gel electrophoresis
SSC:	150 mM NaCl, 15 mM sodium citrate, pH 7.0
ss-cDNA:	single-stranded cDNA
ssDNA:	single stranded DNA
SV40:	Simian Virus 40
TBE:	89 mM Tris-borate, 89 mM boric acid, and 2 mM EDTA pH 8.0
TBS:	Tris-buffered saline
T-TBS:	Tris-buffered saline plus 0.05% Tween 20
TCA:	trichloroacetic acid
TdT:	calf thymus terminal deoxynucleotide transferase
TE:	10 mM Tris-HCl, 1 mM EDTA
TES:	20 mM Tris-HCl pH 8.0, 10 mM NaCl, and 0.1 mM EDTA
TFA:	trifluoroacetic acid
TFP:	trifluopreazine, a calmodulin inhibitor

TM:	tunicamycin, a specific inhibitor for N-glycosylation
2-ME:	2-mercapto-ethanol
Tris-HCl:	tris (hydroxymethyl) aminomethane hydrochloride
TXA_2:	thromboxane A_2
V8:	Staphylococcus aureus protease V8
VLA:	very late antigen
Vnx:	vandyl sulfate-nucleoside complexes, an RNase inhibitor
vWF:	von Willebrand factor
X-gal:	5-bromo-4 chloro-3-indoly-β-D-galactopyranoside

CHAPTER I

INTRODUCTION

A. AN OVERVIEW OF THE CD9 ANTIGEN

Living cells proliferate and/or differentiate in response to signals generated as a result of the interaction between various external stimulants and cellular receptor-like components. Excluding the receptors for hydrophobic molecules such as steroids and thyroid hormones, most cellular receptor-like molecules involved in signal transduction are located in the cell membrane from which an exogenous stimulus is delivered to the cell nucleus through intra-membrane and cytoplasmic signal transducing systems (Yamamoto and Alberts, 1976; Carpenter and Cohen, 1979; Jensen et al., 1982; Evans, 1988). The cell surface antigen CD9 appears to belong to a novel type of such membrane signal-generating molecules. Although first described as a human pre-B cell differentiation marker, it was later found to be present on a wide range of non-lymphoid tissues of both human and non-human origin, as well as on a majority of carcinomas (Jones et al., 1982; Komada et al., 1983; Platt et al., 1983; Dowell et al., 1984; and Seehafer et al., 1984a). The CD9 antigen is a glycoprotein derived from a 20.5 kDa polypeptide precursor which gives rise to a major component of 22-24 kDa (gp22) and a minor component of 24-26 kDa (gp24). Both components are O-glycosylated while the minor one is additionally N-glycosylated. Peptide mapping of the native protein indicates that the different CD9 components share a very similar, if not identical backbone (Newman et la., 1982; Seehafer et al., 1984b; LeBien et al., 1985; and Seehafer et al., 1988). The CD9 antigen is distinguished from most other cell surface glycoproteins by the lack of a transmembrane domain and appears to be anchored to the plasma membrane by covalently bound, long chain fatty acid ligands (Newman et al., 1982). The widespread distribution of the CD9 antigen suggests that it performs a very basic cellular

function, the exact nature of which remains to be elucidated. However, growing evidence supports the notion that this novel surface glycoprotein may participate in cell-environment interaction and in membrane-mediated intracellular signal transduction. Indeed, antibodies to CD9 have been shown to cause platelets to aggregate (Boucheix et al., 1983), fibroblasts to contract a fibrin clot more effectively (Azzarone et al., 1985), and T cells to better respond to mitogen stimulation (Briggs et al., 1987). Purified CD9 protein has also been demonstrated to induce thymidine uptake by antigen producing tumor cells (Zeleznik et al., 1987). However, most of our understanding of the role played by CD9 in cell-environment interaction comes from the study of platelets. Many reports indicate that the CD9 glycoprotein triggers platelet aggregation through a Ca^{++}-dependent event, possibly by interacting with membrane receptors involved in adhesion (Boucheix et al., 1983; Higashihara et al., 1985; Miller et al., 1986; Rendu et al., 1987; Yip and Shaw, 1988; and Slupsky et al., submitted).

B. CD9 AS A PRE-B CELL DIFFERENTIATION MARKER

The ability to establish malignant cell lines from cancer patients represents a major achievement in cancer research. The availability of these cell lines has permitted a more thorough study of the genetics, biochemistry and immunology of malignant diseases. For tumor immunologists, they have provided an inexhaustible source of cells for studying tumor immunologic markers, and for raising antibodies. This in turn has led to the characterization of a number of tumor antigens important to the diagnosis, classification, prognosis, and more recently to the treatment of certain malignancies (Lango, 1987). Furthermore, cell lines from hemopoietic tumors have served as valuable models of normal cell differentiation, since neoplastic hemopoietic cells are generally considered to be the malignant counterparts of immature normal cells (Sachs, 1985, 1986). Differentiation of stem cells along different pathways leading to the mature

functional cell type is accompanied by changes in the cell surface phenotype, which can be used to trace differentiation pathways and identify particular stages of maturation.

In parallel with the success in establishing cell lines was the development of hybridoma technology (Kohler et al., 1975; Milstein, 1980). The technique made it possible to obtain a uniform (i.e. monoclonal) population of antibody molecules, each with an identical antigen-binding site, from a permanent source of antibody-secreting B-cell hybridomas. More importantly, even unpurified molecules such as tumor antigens that constitute only a minor component of a complex cell mixture could be used to generate monoclonal antibodies (mAbs), which in turn can be used as specific probes to localize the antigens, and determine their relative specificity for particular tumors. Furthermore, the mAbs can also be used to purify the antigens in order to study their structure and function.

Kersey et al. (1981) first reported the generation of a monoclonal antibody that recognized a human leukemia-associated and lymphohematopoietic progenitor cell surface antigen. In their study, mAbs were raised against NALM-6-M1 cells, a cell line with a pre-B cell phenotype which was established from acute lymphoblastic leukemic (ALL) cells. Screening the mAbs using both normal lymphohematopoietic cells and fresh, non-cultured, leukemic cells revealed that one particular monoclonal antibody (BA-2, an antibody of the IgG_3 subclass) bound to non-T, non-B ALL cells (mostly pre-B cells) with relatively high specificity. The mAb, however, did not bind to erythrocytes, granulocytes or mononucleocytes, and only bound weakly to T ALL cells (T cell phenotype) and B cell chronic lymphoblastic leukemia cells (CLL, mature B cell phenotype). Immunoprecipitation of [^{35}S] methionine-labeled and surface ^{125}I-labeled cell lysates by the BA-2 mAb and subsequent SDS-PAGE analysis identified the recognized antigen as a polypeptide of 24,000 daltons (p24). Since the antigen was selectively expressed on the surface of ALL cells with cytoplasmic immunoglobulin and

other pre-B cell lineage characteristics, it was suggested that p24 might represent a surface structure present on lymphopoietic bone marrow progenitor cells, or a pre-B cell marker. Subsequent work by the same group (LeBien et al., 1982) using 67 human leukemia/lymphoma cell lines confirmed its initial report. More interestingly, they also showed (Ash et al., 1982) that normal human pluripotential and committed hematopoietic progenitors did not express p24, since complement-dependent cytotoxicity with BA-2 and rosette-separation with the antibody indirectly coupled to ox red blood cells both failed to reduce the number of hematopoietic colonies produced by treated marrow cells. This study clearly established p24 as a surface marker specific for progenitors of the B cell lineage, unless the progenitor cells possess antigens inaccessible to antibody-complement interactions, or are resistant to lysis because of low surface antigen density. However, they also observed that p24 was expressed by many tissues other than lymphoid cells. Following the initial observation by Kersey et al., (1981) several monoclonal antibodies directed against the same antigens were developed and reported independently by different groups (Jones et al., 1982; Maclean et al., 1982; Komada et al., 1983; and Boucheix et al., 1985).

C. WIDESPREAD EXPRESSION OF CD9 ANTIGEN

1. *CD9 Reactive Monoclonal Antibodies*

Through the efforts of hundreds of laboratories worldwide to produce and characterize monoclonal antibodies directed against lymphoid and myeloid antigens, an extensive panel of reagents has become available with which to investigate both normal and neoplastic hematopoietic differentiation. These monoclonal antibodies were extensively characterized over the past 6 years at the Paris (1982), Boston (1984), and Oxford (1986) International Workshops on Human Leukocyte Differentiation Antigens. Monoclonal antibodies defining unique cell surface antigens expressed on lymphoid and

myeloid cells have been segregated into "cluster designation" (CD, a WHO approved nomenclature) groups in an effort to facilitate classification of both mAbs and antigens. It became clear at the First Workshop that the pre-B cell differentiation antigen p24 defined by BA-2 was also recognized by several other mAbs, which were grouped into cluster 9 and the antigen they recognize was termed CD9. However, due to the existence of large numbers of monoclonal antibodies defining the leukocyte differentiation antigen, still more monoclonal antibodies that recognized the CD9 antigen were never submitted. These mAbs were generated against fresh ALL cells and cell lines, non-lymphoid tumor cells, and platelets. Several criteria were used to determine whether a particular mAb could recognize CD9 antigen. These included i) tissue distribution: the antigen recognized by the mAb should display a tissue distribution identical with that of CD9, ii) molecular weight: the polypeptide immunoprecipitated by the mAb should possess a molecular weight of about 24,000, iii) cross-reactivity: if the mAb recognizes the same epitope defined by mAb BA-2, it should inhibit BA-2 binding in sequential immunoprecipitation reactions, or the mAb should compete with BA-2 in binding target cells, iv) dose-response: all the anti-CD9 mAbs have been shown to be able to to induce platelet aggregation (see section F5 for further discussion), and they display a characteristic dose-response curve consisting of an initial lag phase, a single wave profile and a rapid decline of antibody aggregation potency at concentrations below 10 μg/ml; the putative CD9 mAb should display a similar dose-response profile in a platelet activation assay, and finally, v) peptide mapping: the antigen recognized by the mAb should have a proteolytic pattern identical to that of CD9 antigen. More than 10 different monoclonal antibodies have now been reported, which meet some or all of these criteria and which are considered as anti-CD9 mAbs. For example, mAb DU-ALL-1 (Jones et al., 1982) generated against fresh common acute lymphoblastic leukemia (cALL) cells was reported to recognize CD9, since BA-2 and DU-ALL-1 antigens had identical

cellular distributions and showed identical mobilities in SDS-PAGE (Dowell et al., 1984); mAb SJ-9A4 (Komada et al., 1983) raised against cALL cell line NALM-1 and NALM-1-6 cells was thought to recognize CD9 because it demonstrated cross-reactivity with mAb BA-2 and mAb DU-ALL-1 in a target binding assay; and mAb 50H.19 (Maclean et al., 1982) produced against a melanoma cell line MEL-T-1 was believed to react with CD9 as it displayed an identical tissue distribution, immunoprecipitated the same gp22 and gp24 proteins, cross-reacted with BA-2 in immunoprecipitation assays (Seehafer et al., 1988), and produced banding pattern and sensitivity to 2-mercapto-ethanol (2-ME) identical to that of mAb BA-2 and ALB6 upon Western blotting (our unpublished data). mAb ALB6 is another anti-CD9 mAb raised against fresh cALL cells (Boucheix et al., 1985). Other monoclonal antibodies believed to react with CD9 include: i) mAb J2, which was raised to fresh ALL cells (Hercend et al., 1981), ii) mAb FMC 8 (Brooks et al., 1982), mAb FMC 48 (Zola et al., 1984), and mAb FMC 56 (Gorman et al., 1985), which were raised to a pre-B ALL cell line NALM-6, iii) mAb CALL1 (Deng et al., 1983), raised to human schwannoma tissue, and iv) mAb TP82 (Higashihara et al., 1985), mAb AG-1 (Miller et al., 1986), and mAb PMA2 (Hato et al., 1988), raised to washed platelets. mAbs recognizing CD9 are summarized in Table 1. In addition, several other mAbs submitted to the three International Workshops on Leukocyte Differentiation Antigens were found to belong to the CD9 cluster and are also included in the table. However, since most of these mAbs were only presented either as abstracts or as "unpublished observations" in some reports, further information was not available through a literature search. Nonetheless, the fact that diversified materials were utilized to generate these CD9 reactive monoclonal antibodies is strongly suggestive that CD9 antigen is widely expressed on different tissues.

TABLE 1

SUMMARY OF THE MONOCLONAL ANTIBODIES DEFINING CD9 ANTIGEN

Monoclonal antibody	Immunogen	Contributing group	Reference
BA-2	NALM-6-M1	LeBien	Kersey et al., 1981
J2	fresh ALL cells	Ritz	Hercend et al., 1981
DU-ALL-1	fresh cALL cells	Metzgar	Jones et al., 1982
50H.19	MEL-T-1	Longenecker	Maclean et al., 1982
FMC 8	NALM-6	Zola	Brooks et al., 1982
CALL1	schwannoma tissue	Deng	Deng et al., 1983
SJ-9A4	NALM-1/NALM-16	Peiper	Komada et al., 1983a
FMC 48	NALM-6	Zola	Zola et al., 1984
ALB6	fresh cALL cells	Boucheix	Boucheix et al., 1985
FMC 56	NALM-6	Zola	Gorman et al., 1985
TP82	washed platelet	Higashihara	Higashihara et al., 1985
AG-1	washed platelet	Miller	Miller et al., 1986
PMA-2	washed platelet	Hato	Hato et al., 1988
3A9, 3A8, 2E7		Clark	Clark and Yokochi, 1984
602/29*		Boucheix	Boucheix et al., 1985
WB 3*		Deng	Bernard et al., 1984
J30*		Ritz	Reinherz et al., 1986
CLB-thrombo2*		Von de Borne	Reinherz et al., 1986
21D-10*		Garrido	Reinherz et al., 1986
SYB1*		Breard	McMichael et al., 1987
ML13*		Rieber	McMichael et al., 1987
J9*		Pesando	McMichael et al., 1987
BA1*		LeBien	McMichael et al., 1987
BU-16*			McMichael et al., 1987

*mAbs believed to belong to CD9 cluster, but cannot be verified through literature search are also included. Most of these antibodies were only described in the abstracts and/or tables of the First, Second and Third International Workshops on Human Leukocyte Differentiation Antigens.

NALM-6, NALM-6-M1, NALM-1 and NALM-16 are cALL cell lines, and MEL-T-1 is a melanoma cell line.

2. *Tissue Distribution of the CD9 Antigen*

The wide distribution of CD9 antigen is reflected by its presence on tissues originating from the endoderm, mesoderm, and ectoderm, in contrast to most other CD antigens which are restricted to lymphoid tissues. CD9 represents one of the three major surface proteins on the platelet plasma membrane (Seehafer et al., 1988), and is highly expressed on smooth muscle, cardiac muscle, vascular endothelium, epithelial cells of distal renal tubules and Bowman's capsules, thyroid, mammary duct, intestinal mucosal membrane, tonsillar crypts, salivary gland, pulmonary alveoli, Hassal's corpuscles (thymus), red pulp of spleen, and glial cells (Jones et al., 1982; Komada et al., 1983; Platt et al., 1983). It is also weakly expressed on normal human fibroblasts (Boucheix et al., 1985a). In addition, and in contrast to earlier reports, the CD9 antigen was located on hemopoietic cells other than pre-B cells, such as activated T-cells (Hercend et al., 1981), activated B-cells (Hsu et al., 1984), granulocytes and monocytes (Brooks et al., 1982), and eosinophils (Ohto et al., 1987). Moreover, CD9 antigen was reported to be present on platelets and distal renal tubules from such lower primates as the chimpanzee, orangutan, mangaby and rhesus monkey, as well as non-primates such as the rabbit (Dowell et al., 1984), the sheep, and the dog (our unpublished observations). Normally, the function of a particular protein is reflected by its tissue distribution, since each tissue performs a characteristic function. The ubiquitous presence of CD9 discriminates it from the majority of non-MHC antigens, suggesting that it serves a fundamental cellular function. Although this wide tissue distribution of CD9 may make it difficult to uncover its specific function(s), closer analysis reveals a discernible pattern: CD9 is primarily expressed on excitable, secretory, and proliferating tissues, suggesting that it is involved in the processes of cellular signal transduction originating from the cell membrane, a very basic cellular function essential for cell-environment interaction. The fact that CD9

is expressed on smooth muscle and gliocytes, but not on skeletal muscle and neurons (Jones et al., 1982) suggests that CD9 transduces the signal through ligand binding instead of through action-potential generation across the plasma membrane. In addition, one of the obvious similarities between skeletal and neuronal tissues is the inability to regenerate after tissue damage, whereas smooth muscle and gliocytes possess considerable regenerative capabilities, suggesting that CD9 expression in these cell types might also be related to proliferative as well as regenerative potentials. This apparent association of CD9 with cell proliferation is further illustrated by its presence on tumor cells.

D. CD9 AS A TUMOR-ASSOCIATED ANTIGEN

CD9 antigen was first characterized by a monoclonal antibody developed to ALL cells. Its presence, therefore, may be expected on tumors from lymphoid tissues. Indeed, in the first report on CD9 antigen, Kersey et al. (1981) observed that although CD9 appeared to be restricted to B-cell progenitors and B-cell leukemia (non-T, non-B ALL), it was also present on some malignant T-cells (T ALL). Subsequent reports extended the observed distribution of CD9 to Burkitt's lymphoma (BL), non-Hodgkin's lymphoma (NHL), chronic myelocytic leukemia in blast crisis (LeBien et al., 1982; Brooks et al., 1982), and acute non-lymphoblastic leukemia (ANLL, Ashman et al., 1987). Unexpectedly, CD9 expression was frequently observed in tumors from non-hemopoietic tissues. It was reported (Seehafer et al., 1984a) to be strongly expressed in human cell lines derived from tumors of bone, colon, kidney, cervix, pancreas, skin, breast, as well as placenta and in biopsy materials from tumors of human colon, breast, kidney, and lung. In fact, a panel of thirteen carcinoma cell lines examined tested positive for CD9 expression. In addition to being a pre-B cell marker, the widespread expression on solid tumors also qualified the CD9 as a tumor-associated antigen. The

existence of CD9 antigen on tumors other than hemopoietic malignancies such as neuroblastoma and retinoblastoma was later reported (Boucheix et al., 1985) along with the observation that the frequency of CD9 expression in tumors is much greater than that found in normal tissues, suggesting that the antigen is present on the transformed progeny of antigen negative normal tissues. This notion is derived from the fact that the antigen exists on biopsy materials and cell lines from hepatoma and neuroblastoma, while hepatocytes and neuronal cells totally lack CD9 expression (Jones et al., 1983; Seehafer et al., 1984a; Boucheix et al., 1985; our unpublished data). Since tumor cells may be considered as the transformed progenitors of mature normal cells which are arrested at particular stages of differentiation, the ubiquitous distribution of CD9 antigen in tumors might be explained by proposing that it is expressed in rapidly proliferating progenitor cells and that some mature progeny cells retain its expression, while others lose it. This proposal is supported by several studies of CD9 expression on renal tubules, and on renal carcinomas. CD9 is expressed only on adult distal tubular epithelia, not on adult proximal tubular epithelia (Jones et al., 1982; Platt et al., 1983). However, CD9 is strongly expressed on renal epithelial tumors of presumed proximal tubule origin, and Wilm's tumors (a more primitive tumor that derives from the kidney stem cells) are always positive for CD9, even though they lack markers for proximal tubule epithelium (Borowitz et al., 1986). Since CD9 expression is manifested early and is subsequently lost in proximal regions during renal tubule ontogenesis (Platt et al., 1983), its presence in renal tumors could represent the re-expression of a fetal differentiation antigen. The fact that CD9 is highly expressed on rapidly growing fetal fibroblasts while it is only weakly expressed on slower proliferating adult fibroblasts (Seehafer et al., 1984), and that it is expressed by activated T, and B cells, and not by their terminally differentiated, or resting counterparts (Kersey et al., 1981; Hercend et al., 1981; Hsu et al., 1984) also supported its possible involvement during

differentiation, and its association with the state of proliferation. Indeed, after infection of adult fibroblasts with SV40 virus, a procedure that is believed to be able to alter the growth characteristics of the recipient cells, the CD9 antigen became highly expressed (our unpublished data). In addition, it was recently observed that the antigen purified from platelet lysate through mAb (DU-ALL-1) affinity chromatography was mitogenic for tumor cells bearing CD9 protein (Zeleznik, et al. 1987). Nanogram quantities of the protein were found to induce thymidine uptake by an adherent pancreatic carcinoma cell line HPAF-2, which expressed CD9. Cells that did not express the CD9 were not stimulated by the same treatment (Zeleznik, et al. 1987). However, due to the lack of information on CD9 function, interpretation of the mechanism(s) involved must await further study. Finally, the apparent association of CD9 with tumor cells and the similarities of its tissue distribution, chromosome location and molecular weight to p21/ras, an onco-protein involved in cellular transformation, prompted the experiments designed to test if the two proteins were structurally and/or functionally related (LeBien et al., 1985). The result of the study demonstrated that CD9 was indeed a novel tumor-associated antigen which was not related to p21/ras. At the present time, the role played by the CD9 in cell proliferation and oncogenesis is still poorly understood. A better understanding of the biological function of CD9 can only be achieved through a thorough study of its biochemical structure, and its relationship with other proteins.

E. BIOCHEMICAL CHARACTERIZATION OF CD9

1. *Three Glycosylated Components*

CD9 antigen immunoprecipitated from cALL cell line NALM-6-M1 was first reported to be a polypeptide with a molecular weight of 24,000 (Kersey et al., 1981), or 26,000 (Hercend et al., 1981; Deng et al., 1983), and later observed to be composed of a major component of 22 kDa and a minor component of 24 kDa (Seehafer et al., 1984;

LeBien et al., 1985). The discrepancy in the molecular weight estimation by different groups may be explained by their use of different molecular weight markers and conditions for SDS-PAGE. For the sake of convenience in the following discussion, the major component will be assigned a molecular weight of 22,000, and the minor component 24,000. The major component contains O-glycosidically linked oligosaccharides, since pulse-chase labelling of p22 showed a post-translational modification resulting in a molecular weight increase of approximate 500-1000, and alkaline treatment resulted in a decrease in molecular weight of the same amount. On the other hand, it does not seem to contain N-asparagine linked oligosaccharides, because treatment with glycosidase or exposure of cells to tunicamycin (TM) failed to show any change in its molecular weight. In addition, it failed to bind to lectin affinity columns of concanavalin A (conA), lentil lectin or *Ricinus communis* lectin (Newman et al., 1982; Jones et al., 1982). The glycoprotein nature of CD9 (gp22) was confirmed by Komada et al. (1983), using a solid phase indirect radioimmunometric assay. In addition, they observed a difference in the glycosylation of gp22 from cALL and neuroblastoma. Unequivocal evidence for the glycosylation of CD9 polypeptide came from a thorough biochemical study by Seehafer et al. (1984b). The major component (gp22) was shown to be O-glycosylated following incorporation of ^{3}H-galactose and resistance to TM. Furthermore, the minor component (gp24) was shown to be both O-glycosylated and N-glycosylated, the N-glycosylation being demonstrated by incorporation of ^{3}H-mannose and by the total inhibition of its synthesis in the presence of TM. Interestingly, both components of CD9 appeared to be derived from a common 20.5 kDa precursor, since only a 20.5 kDa component was detected from the antigen producing cells pulse-labeled for 5 minutes in the presence of TM. The fact that mAb 50H.19 used in the study recognized the glycoprotein precursor also indicated that the antibody was directed at the CD9 peptide back-bone, not to the carbohydrates attached to it. The difference in

glycosylation between gp22 and gp24 was later confirmed by pulse-chase analysis, and by digestion with the N-linked carbohydrate-specific endoglycosidase-F (LeBien et al., 1985). The relationship between the 20.5 kDa precursor and the gp22/gp24 proteins was also verified by peptide mapping, whereupon gp22 and gp24 were shown to produce very similar protease cleavage patterns (LeBien et al., 1985; Seehafer et al., 1988). Recently, an additional minor component of CD9 with a molecular weight of 27,000 (gp27) was detected in platelets (Miller et al., 1986; Seehafer et al., 1988). The cleavage pattern obtained by the limited proteolysis indicated that it is also derived from the common precursor of gp22 and gp24. It would seem, therefore, that the expression of different components of the CD9 antigen might follow a particular pattern in different tissues. Indeed, our laboratory has observed that although activated T cells and Vero cells (an African green monkey kidney cell line) possess only the major component of gp22, most other CD9 producing cells express both the major gp22 and the minor gp24, while platelets elaborate an additional minor gp27 (Seehafer et al. 1988a). This apparent tissue-specific glycosylation pattern of CD9 antigen expression may not be a random process.

2. *Secondary Structure Involves Disulfide Bonds*

Contrary to the earlier report of Newman et al. (1982), all CD9 components were later shown to contain intra-chain disulfide bonds, which were responsible for bringing about an apparent increase of molecular weight of about 500 when analyzed by SDS-PAGE under reducing conditions (Seehafer et al., 1984b). In addition, since each of the reduced components underwent a similar molecular change upon SDS-PAGE, Seehafer et al. (1984b) suggested that they contained a similar degree of intra-chain disulfide bonding, a finding consistent with the fact that the components seemed to be derived from a common precursor. The existence of disulfide bonds in these molecules was further supported by the demonstration that the mAbs 50H.19, BA-2 and ALB-6 failed

to recognize CD9 on Western blots if the antigen was reduced prior to SDS-PAGE. These observations were later confirmed by Miller et al. (1986), who reported an increase in electrophoretic mobility upon reduction of ^{125}I-labeled CD9 protein immunoprecipitated from platelets with mAb AG-1. Hato et al. (1988) also noted that mAb PMA2 no longer recognized CD9 on Western Blots once the antigen was reduced.

3. *Attachment to Membranes via Fatty Acids*

Despite the lack of the amino acid sequence of the peptide, the CD9 antigen was considered a non-integral membrane protein because it could not be labeled by the lipophilic photoactivatable nitrene reagent, ^{125}I-labeled hexanoyldiiodo-N-(4-azido-2-nitrophenyl)tyramine (Newman et al., 1982). This lipophilic nitrene reagent was shown to allow cell membrane components to be labeled according to their hydrophobicity, and was used to determine whether a membrane protein was integral in nature (Owen et al., 1980). It was demonstrated, for example, that HLA-ABC, immunoglobulin heavy chains, and α and β chains of HLA-DR were labeled by the reagent, whereas β_2 microglobulin and immunoglobulin light chains were not (Owen et al., 1980).

CD9 differs from most other cell surface glycoproteins in that it lacks a transmembrane domain, which suggests that the antigen may be released by the antigen producing cells. Indeed, antigen shedding was initially detected from cALL and neuroblastoma cell lines using a solid phase indirect radioimmunometric assay and immunoadsorption of CD9 in spent medium of metabolically labeled cells (Komada et al., 1983). Furthermore, the antigen was detected in the fractionated plasma of five patients with cALL at the time of diagnosis and was undetectable when the patients had achieved a complete remission. However, earlier pulse-chase labelling experiments on NALM-1 and NALM-6 (both are high antigen producing cell lines) failed to show the release of the antigen into the culture media up to 5 hours into the chase (Newman et al., 1982). Finally, experiments from our laboratory designed to address the same issue with

the spent media of NALM-6 cells metabolically labeled with both [^{35}S] methionine and/or [^{3}H] leucine did not detect any antigen shedding. Similar results were obtained with several other antigen producing cell lines, thereby excluding the possibility that the shedding might be tissue or cell line dependent (our unpublished data). Moreover, the protein was observed to have a half-life in excess of 40 hours, suggesting that it is firmly anchored in the plasma membrane. It would seem, therefore, that the shedding process reported by Komada et al. (1983) may be caused by the breakdown of the antigen-bearing cells or tissues. In fact it has been reported that CD9 antigen can be immunoprecipitated from the spent media of overgrown cell cultures (Newman et al., 1982).

The wide occurrence of acylation as a posttranslational modification of membrane proteins has only been recognized within the last 10 years. In turn, an increasing number of prokaryotic and eukaryotic membrane proteins have recently been found to contain covalently bound long chain fatty acids (Schmidt, 1983). In most cases, the fatty acid involved is the 16-carbon saturated fatty acid palmitic acid, and more rarely the 14-carbon saturated fatty acid myristic acid (Buss and Sefton, 1985). The protein-bound fatty acids are proposed to serve in the transport of glycoproteins from the Golgi complex and in anchoring to the plasma membrane (Schmidt, 1983; Cross et al., 1987; Ferguson and Williams, 1988).

Although the firm attachment of CD9 to plasma membrane may be difficult to model considering that the protein does not possess a transmembrane domain and that it does not form detectable complexes with other proteins which might attach it to the plasma membrane (Newman et al., 1982; Seehafer et al., 1984), this paradox may be explained by the findings that CD9 contains long chain fatty acids. Following intrinsic labelling with [^{3}H] palmitic acid and SDS-PAGE, the immunoprecipitated antigen showed extensive incorporation of palmitic acid in both gp22 and gp24 components

(Seehafer et al., 1985a). Similar results were obtained by LeBien et al. (1985) who reported that palmitic acid was covalently attached to gp22 and gp24. It was also recently reported that CD9 is the major fatty acid acylated surface component of human platelets (Seehafer et al., 1988), and that all three components of CD9 are extensively acylated.

The non-integral nature of the CD9 protein would prevent it from recycling to subcellular sites for acylation. Since platelets lack protein synthesis capacity, the palmitylation of CD9 is thought to be mediated by a plasma-membrane associated transacylase which functions independently of protein synthesis. Treatment of the SDS gel containing the [^{3}H] palmitic acid-labeled CD9 protein with hydroxylamine, a compound that is nucleophilic for ester linkages, led to an almost complete loss of label in gp22, suggesting that the fatty acids are attached to the protein via an ester linkage (LeBien et al., 1985). This was also confirmed independently by the inability of chloroform/methanol extraction (which partitions non-covalently bound lipid) to remove the radiolabel from the [^{3}H] palmitic acid-modified CD9, and by hydroxylamine treatment of the similarly labeled antigen in the form of immunocomplexes (Seehafer et al., 1988). More recently, it was observed that CD9 contains multiple fatty acid ligands, as demonstrated by localization of the fatty acid incorporation into two peptides generated from the intact antigen, and by the apparent decrease of molecular weight of 1,000 in one of the peptides following hydroxylamine treatment and the increase of the same mass attributable to palmitic acid (with a molecular weight of about 250) ligation (Seehafer et al., 1988). This finding distinguishes CD9 from other externally located non-integral glycoproteins, which have only one membrane attachment site. Interestingly the multiple acylation sites were localized to a membrane proximal peptide, a presumed functional region that contains the binding site for mAb 50H.19. The fact that CD9 is both variably, and reversibly acylated at multiple acylation sites within this functional region suggests that acylation may play a role in anchoring the protein to the plasma

membrane as well as in modulating its interaction with other membrane proteins (Seehafer et al., 1988).

4. *Associated Protein Kinase Activity*

Membrane protein kinases have received much attention as a consequence of the discovery of tyrosine phosphorylating enzymes associated with receptors for growth factors and with oncogene products (Hunter, 1987). It was therefore very interesting to observe that the immunocomplexed CD9 proteins (already recognized as being linked to the malignant phenotype; see Section D) exhibited protein kinase activities (Seehafer et al., 1984). Immunocomplexes formed after incubating the mAb 50H.19 (attached to protein A Sepharose beads) with CD9 producing tumor cell lysates were able to phosphorylate casein and phosvitin in a specific manner, suggesting that the associated protein kinase phosphorylates on serine and threonine, but not tyrosine. The enzyme activity is cyclic AMP-independent. Recently a protein kinase activity co-precipitating with CD9 antigen was also reported in a separate study (Zeleznik et al., 1987). CD9 isolated from platelet lysates by mAb DU-ALL-1 affinity chromatography was demonstrated to possess protein kinase activity with the same substrate specificity as that described by Seehafer et al. (1984). However, neither study could determine whether the kinase activity was intrinsic to the CD9 protein because several proteins were co-precipitated by the mAbs. In fact, the lack of the transmembrane domain and the small molecular mass of CD9 would strongly argue against this possibility. Indeed, when the mAb affinity purified protein was further purified by electro-elution following SDS-PAGE, it no longer displayed any kinase activity (our unpublished data). However, the demonstration of CD9 immunocomplex-associated protein kinase activity may indicate that CD9 activates a kinase indirectly. Phosphorylation may then serve to transduce the signal from CD9 to the next substrate. The identification of a phosphorylated 110 kDa glycoprotein in tumor cells which co-precipitates with CD9 lends support to this notion

(Seehafer et al. 1984a). Whether the kinase activity detected in CD9 immunoprecipitates is the property of a physically associated kinase is not known. Finally, it is also interesting to note that the kinases co-precipitated with CD9 from both tumor cells and platelets share the same substrate specificity, suggesting that the signal transduction by CD9 in platelets and malignant cells may involve a common protein kinase, and possibly similar pathways.

5. *Chromosomal Location of the CD9 Gene*

When living cells are treated with certain inactivated viruses or polyethylene glycol, reagents that alter the biological properties of the plasma membrane, they tend to fuse with each other to form a large cell with two separate nuclei (heterokaryon). Eventually, the heterokaryon proceeds through mitosis and produces a hybrid cell in which the separate nuclei combine to form a single nucleus (Harris and Watkins, 1965). For unknown reasons, hybrids produced between human and rodent cells are unstable and tend to lose human chromosomes, giving rise to a variety of different mouse-human or hamster-human hybrid cell lines, each of which contains only one or a few human chromosomes. Most of these human chromosomes still retain their function, even though they are maintained in a different context. Analysis of many of these hybrid cell lines has made it possible to assign particular biochemical functions to particular human chromosomes (Ruddle and Creagan, 1975).

CD9 antigen detectable by murine mAb has not been found on either mouse or hamster cells (Dowell et al., 1984). Therefore, CD9 antigen detected by mAbs on the surface of human-mouse or human-hamster hybrid cells are expected to be expressed by the human chromosomes residing in the hybrids. An analysis of 20 independent human-mouse and human-hamster hybrid cell lines enabled Boucheix et al. (1985) to demonstrate that the gene coding for CD9 is located on chromosome 12 by its syntenic correlation with LDH-B, a well known marker for chromosome 12. Furthermore,

expression of the antigen in a hybrid from parental human fibroblasts possessing the balanced reciprocal translocation 46,X,Y,t (X,12) (q23,q12) indicated that the CD9 gene is probably localized on 12q12-->pter.

F. BIOLOGICAL EFFECTS OF CD9

1. *Agonistic mAb and Cell Activation*

The great majority of cell-surface receptors demonstrating affinity for hydrophilic signaling molecules are thought to undergo a conformational change when they bind to a ligand (first messenger) at the cell exterior. This leads to the production of an intracellular signal (second messenger) which alters the behavior of the cell. The ligand-receptor complex can generate this second message by activating or inactivating a plasma-membrane-bound enzyme which, in turn, catalyses the formation of "activating" signals in the form of a soluble intracellular mediator (e.g., cAMP), or modified protein (e.g., phosphorylated proteins). Alternatively, cell-surface receptors may open or close gated ion channels in the plasma membrane, thereby causing either a small and transient flux of ions that briefly changes the voltage across the plasma membrane (as in neurons and skeletal muscles) or a major influx of ions into the cytosol (as in other electrically inactive tissues), to initiate an intracellular response. There is increasing evidence that Ca^{++}, like cAMP, is an important intracellular regulator and that it functions as a second messenger for certain extracellular signaling molecules (Racker, 1980). Many of the electrically inactive cells have surface receptors that are functionally linked to Ca^{++} channels in the plasma membrane, which allow Ca^{++} to enter the cytosol following receptor-ligand binding.

It is now well established that mAbs to a number of cell surface antigens can activate antigen producing cells either by functioning alone ("competence" signals), or in conjunction with other stimuli ("progression" signals; Clark and Ledbetter, 1986).

Termed "agonistic" antibodies, they provide an alternative and complementary approach to study unknown factors in cell activation, since they may mimic membrane signals generated by the interaction between those factors and the antigens. The major advantage of natural factors over agonistic mAbs is that they deliver physiological signals, while an agonistic mAb is presumed only to mimic a physiological ligand. The purified agonistic mAbs are, however, homogeneous and react with antigens which can be readily defined biochemically. Eventually, the role played by the antigens, and hence by the factors in cellular activation can be explored thoroughly, and the other structures that interact with those antigens identified accordingly.

2. *CD9 mAbs are Agonistic Antibodies*

As discussed before, the tissue distribution pattern of CD9 antigen has clearly indicated that it may play a role in membrane-mediated signal transduction events, although at the moment, it is not known how CD9 is normally activated under physiological conditions. Theoretically this can be achieved either through binding to a natural external ligand, or through interacting with an intra-membrane ligand, itself representing an already activated protein upstream in the signal transducing cascade. If CD9 plays a role in cellular interaction with its environment and in cell activation, antibodies to CD9 antigen should, therefore, either inhibit its function by binding to the functional domains to block ligand binding, or enhance its function by mimicking the ligand, resulting in cell activation. Evidence suggesting that the CD9 mAbs are agonistic antibodies comes from studies of CD9 functions in lymphocyte activation (Briggs et al., 1987), fibrin clot retraction (Azzarone et al., 1985), and most significantly, in platelet aggregation (Boucheix et al., 1983). It is worthwhile to point out that most if not all of the CD9 mAbs reported so far have been shown to mimic the activation signal transduced by the antigen. This suggests that although the hydrophobic functional regions of CD9 (containing fatty acids) are usually shielded by the hydrophilic

immunogenic domains, binding of the agonistic antibodies may cause a conformational change in the antigen, which exposes the functional domains. This structural change, in turn, modulates the interaction of the CD9 with other membrane proteins, leading to full signal generation.

3. *Role of CD9 in Lymphocyte Activation*

Cell activation represents an important aspect of the response of lymphocytes to specific antigens or mitogens and results in the acquisition of specific functions. The characterization of antigens appearing on lymphocytes during activation provides an important method of studying the mechanisms involved in lymphocyte response.

Although CD9 was first described as a pre-B cell differentiation marker, few studies if any were designed to investigate its function in B cell activation and/or differentiation. The role played by CD9 in lymphocyte activation is beginning to be understood through experiments carried out mostly on T cells. Hercend et al. (1981) have observed that CD9 is expressed by activated T cells (both T-helper and T-cytotoxic/suppressor) although it is completely absent from the resting cells. Furthermore, the CD9 antigen expression by phytohemagglutinin (PHA), concanavalin A (Con A), or by alloantigen stimulated T cells is believed to be a late event, since kinetics of CD9 expression by cytofluorographic analysis using mAb J2 demonstrated that the antigens occurred only after 3 to 4 days of induction, peaked on day 5-6, and began to decrease on day 10. The inducibility of CD9 in lymphoid tissues was also confirmed by Brooks et al. (1982) who observed an increase in CD9 expression in normal peripheral blood mononuclear cells stimulated with PHA and pokeweed mitogen (PWM) over a period of 8 days in culture, and more recently by Yasukawa et al. (1988), who reported that phorbol-ester (PMA) not only induced CD9 antigen in mature T cells, but also in mature B cells, monocytic cell lines, and pre-T cells, suggesting that CD9 expression is not restricted to any specific cell lineage but rather seems to depend on a

particular stage(s) of cell differentiation. Most interestingly, CD9 expression was found to correlate with the differentiation of the CD9 negative human promyelocytic leukemia cell line HL-60 into adherent cells morphologically determined to become monocytes, suggesting that CD9 antigen may be involved in the cell adhesion process (see next section for further discussion).

It was originally reported (Hercend et al. 1982) that T cell proliferation driven by mitogens, alloantigens, or soluble antigens was not blocked by exposure to the CD9-reactive mAb J2. Generation of cytotoxicity in mixed lymphocyte reactions (MLR) or induction of immunoglobulin secretion in a pokeweed mitogen driven system was not modified by antibody exposure either. However, an inhibitory effect of CD9 antibody has recently been reported by De Rie et al. (1987), who demonstrated the reduction of PMA-driven immunoglobulin synthesis by B cells in the presence of mAb J2, and by us. We observed that the CD9-reactive mAb 50H.19 is inhibitory to MLR and to the stimulation of T cells by PPD-pulsed leukocytes (Masellis-Smith et al., 1988A). In fact on a molar basis mAb 50H.19 is a more effective antagonist than mAbs directed to CD4 or HLA-DR. The discrepancy between the earlier, and later findings may reside in a very critical relationship between dosage, and inhibition.

It was unexpected that CD9 mAb would be inhibitory to T cell activation since CD9 is absent from both resting T and B cells. This apparent paradox was resolved by our observation that the antibody did not act directly on T cells but instead reacted with the accessory cell components to bring about the inhibition, since the inhibition was abolished if the responding cells were first depleted of low-density mononuclear cells on a Percoll gradient (Masellis-Smith et al., 1988).

Recently it was observed that the anti-CD9 mAb J2 actually augmented the lymphoblastic response to PHA in a thymidine incorporation assay measured at 72 hours, using peripheral blood lymphocytes from normal donors, and from B-CLL

patients (Briggs et al. 1987) suggesting that CD9 may additionally regulate T cell proliferation. Lymphocytes are believed to be activated by two separate signals generated by antigen binding and by interaction with collaborating T cells (Bretscher, 1974; Farrar et al., 1982). Why mAb to CD9 promote enhancement of mitogen driven proliferation, but inhibit antigen-driven proliferation may be explained by the relative requirement for accessory cell function in the two systems.

In the PHA stimulation assay, the antibody (second signal) appears to act in conjunction with PHA (first signal, and a soluble mitogen that does not require processing by accessory cells) to enhance the proliferative response. In the MLR assay, the first signal derives from recognition of the alloantigens presented on accessory cells. Presumably the antibody interferes with the ability of accessory cells to initiate T cell activation possibly by blocking the recognition of determinants on CD9 critical for T cell accessory cell interaction. However, in a mitogen-driven response the requirement for accessory cell function is largely by-passed so that T cells activate, and express CD9. Since CD9 initiates intracellular signals it is possible that mAb activate signalling pathways on the activated T cell which synergize with the mitogen activation signals.

4. *Role of CD9 in Cell Adhesion*

The ability of a cell to extend, adhere and contract is the universal basis of morphogenesis, a process by which different cell types arrange themselves in a precisely organized pattern, and give the body structure a well-defined shape. This cell movement is mediated by the interaction between the cellular membrane components and the extracellular matrix (Thiery et al., 1985; Buck and Horwitz, 1987). In addition, groups of cells are able to obtain their positional information through membrane structures in order to coordinate their growth and differentiation by expressing a particular set of genes so that they can stay together and remain distinct from the cells of surrounding tissues (Bissell et al., 1982; Hay, 1984; Menko and Boettiger, 1987). Some sponge

cells, for example, adhere to cells of their own species by secreting large, species-specific aggregation factors that cross-link similar cells together (Humphreys et al., 1977). Cells dissociated from various tissues of vertebrate embryos preferentially associate with cells from the same tissue when they are mixed together (Moscona and Hausman, 1977). Normal mammalian cells grown in culture will stop dividing after they form a confluent monolayer, a phenomenon known as contact inhibition of cell division (Folkman and Moscona, 1978). Tumor cells, on the other hand, seem to have lost the ability to effectively communicate with their immediate environment. They no longer retain a regular tissue architecture, they continue to grow and pile up on top of each other even after they have formed a confluent monolayer, and they become detached from the site of origin and metastasize to distant sites within the organism.

The recent isolation and characterization of a family of structurally related receptors involved in cell adhesion, cell migration during embryogenesis, platelet aggregation, lymphocyte help and killing, and phagocytosis have shed light on the molecular mechanisms underlying the process of cell adhesion (Hynes, 1987). Collectively designated as integrins, these molecules consist of a heterodimer of two non-covalently linked subunits, a 95-130 kDa homologous β-chain and a 130-210 kDa heterologous α-chain, and recognize a tripeptide arg-gly-asp (RGD) sequence which is common to many extracellular ligands and which is thought to play a key role in cell adhesion (Ruoslahti and Pierschbacher, 1986). The binding specificity of each receptor to its ligand is believed to be influenced by the critical domains of the ligand separated from the RGD sequence and by the interaction of the receptor with other matrix components, as is indicated from the studies on the most extensively characterized cell adhesive protein, fibronectin (Chiquet-Ehrismann et al., 1988; Obara et al., 1988; Ruoslahti, 1988).

The evidence for the involvement of CD9 in cell adhesion came from the demonstration of clot retraction by fibroblasts (Azzarone et al., 1985). Normal

fibroblasts cultured *in vitro* are able to spread within fibrin and to induce the retraction of a fibrin clot. The efficiency of this fibrin clot retractile (FCR) activity is dependent on the growth phase; it is maximal during exponential growth and reduced in post-confluent cultures. Furthermore, it seems to be correlated with some aspects of cell transformation *in vitro*, since FCR efficiency is reduced in established nontumorigenic cell lines and is lost in tumorigenic cancer cell lines (Azzarone et al., 1983). Interestingly, it was later observed that treatment of normal fibroblasts with the CD9-specific mAb ALB6 was able to cause a significant increase in the FCR efficiency in post-confluent cells indicating an enhance ability to connect the contractile elements of the cytoskeleton to the extracellular matrix through cell-adhesion receptors (Azzarone et al., 1985). However, no effect on the FCR ability could be observed by treatment with the same antibody when an osteosarcoma cell line (Te85) was used. This finding suggested that CD9 may be involved either directly in fibroblast binding to fibrin, or in events following such binding. Furthermore, the fact that CD9 mAb could not enhance tumor cells to retract a fibrin clot indicates that the CD9-mediated mechanisms may become non-functional as a result of transformation, even in the presence of the antigen (i.e. the Te85 cell line used in the study was a strong CD9 producer). Another observation supporting this concept is the ability of mAb 50H.19, when added to cultures of leucocytes isolated from normal peripheral blood, to cause the monocytes to adhere to the tissue culture flasks in a specific manner. In addition, when tumor tissue biopsy materials were examined, CD9 antigen was always found more heavily distributed around the margin or surface of the tumor mass (our data), again suggesting that the function of the antigen may be to interact with the surrounding stroma. Finally, the recent observation of Yasukawa et al. (1988) correlating the differentiation of HL-60 cells (a premyelocytic cell line) into adherent monocytes with the expression of the CD9 antigen further supports our hypothesis that CD9 is intimately involved in the process of cell adhesion.

The molecular mechanism for cell adhesion is still poorly defined. In the case of fibroblast-induced fibrin clot retraction, at least several different cell surface structures are involved. Membrane receptors for fibrinogen and fibrin molecules are necessary for attachment to the substratum (Colvin et al., 1979), secretion of cellular factors are involved in the polymerization process (Birck-Bichler et al., 1981), and cytoskeletal filaments are necessary for the expression of cellular spreading and contraction (Azzarone and Macieira, 1984). Since all these processes appear to be calcium dependent, the retraction induced by CD9 mAb may also be mediated by changes in intracellular calcium levels. Indeed, fibrin clot retractile activity was inhibitable in a dose dependent manner by the calcium channel-blocking agents Diltiazem and Verapamil and by the calmodulin (a ubiquitous intra-cellular Ca^{++} binding protein that plays a part in the majority of the Ca^{++}-regulated processes) inhibitor trifluopreazine (TFP, Azzaron et al., 1985). Interestingly, it was also observed that treatment of fibroblasts with the CD9-specific mAb ALB6 reversed the FCR inhibitory effect of the calcium-channel blocking drugs without affecting that of TFP. This indicates that the CD9 mAb target on the cell membrane is probably the same as that of the two calcium-channel blockers. The experiment also suggests that CD9 is active in the regulation of the calcium flux which controls fibrin clot retraction in normal human fibroblasts. The reduced FCR efficiency observed in osteosarcoma cells may be due either to the inability of CD9 to induce the calcium flux, or to alterations in other calcium-mediated events (Azzaron et al., 1985). The former possibility, however, seems less likely because CD9 mAb 50H.19 was shown to cause a calcium flux in ALL cell line NALM-6 in the presence of a second cross-linking antibody (Yip, and Shaw 1988). Cross-linking of CD9 mAb was thought to augment the signals from antigen-antibody binding because calcium flux was inducible in washed normal human platelets only under such conditions.

5. Role of CD9 in Platelet Aggregation

The mechanisms of platelet activation by physiological agonists is still not fully understood, but is crucial to an understanding of CD9 function. Despite its ccmparatively simple structure the platelet is functionally complex. To maintain primary blood hemostasis following vascular injury, rapid formation of platelet plugs at the site of injury must be achieved. This is usually accomplished within minutes following trauma and is of prime importance in stopping blood loss from capillaries, small arterioles, and venules. The primary hemostatic plug is further strengthened by the fibrin strands that are formed in secondary hemostasis during which plasma coagulation takes place. Secondary hemostasis is particularly important in larger vessels and prevents secondary bleeding hours or days after the initial injury.

Effective primary hemostasis requires three critical events: platelet attachment to the surface of the damaged vessel (adhesion), the release of various agonists (granule release) and platelets binding to each other (aggregation). Within a few seconds following injury, platelets start to adhere to collagen fibrils in vascular subendothelium. This adhesion process is facilitated by the von Willebrand factor (vWF), an adhesive glycoprotein which allows platelets to remain attached to the vessel wall despite the high shear forces generated within the vascular lumen. vWF accomplishes this task by forming a link between platelet receptor sites and subendothelial collagen fibrils. Adherence of platelets induces configurational changes in their plasma membranes, thereby altering their shapes and triggering a complex sequence of activation events such as: i) secretion of intracellular components (granule release), including thrombospondin, platelet factor 4 (which is a cationic anti-heparin agent that facilitates platelet binding to vessel walls), vWF, α-thromboglobulin, fibrinogen, and fibronectin, etc. from alpha granules; calcium, ADP, and ATP and serotonin, etc. from the dense granules; and endoglycosidase, etc. from the lysosomes, ii) liberation of the arachidonic acid from

platelet phospholipids (phosphatidylcholine and phosphatidylinositol) by phospholipase A_2 and phospholipase C, thereby providing the substrate for cyclooxygenase to generate thromboxane A_2 (TXA_2), a prostaglandin-dependent process which is inhibitable by aspirin and non-steroidal anti-inflammatory drugs that suppress the cyclooxygenase. The release of both cyclic peroxides and TXA_2 cause further granule release, platelet aggregation and vasoconstriction. By inhibiting cAMP formation, TXA_2 also causes calcium mobilization to promote aggregation, iii) the catalyzation of the interaction between plasma coagulation proteins leading to thrombin formation, and iv) recruitment and aggregation of more platelets. Finally, ADP released from the activated platelets modifies the platelet surface so that fibrinogen can attach to a complex formed between membrane glycoproteins IIb and IIIa and link adjacent platelets into a hemostatic plug. The plug is also strengthened by the released thrombospondin which forms a complex with fibrinogen and thereby stabilizes the platelet aggregates.

Boucheix et al. (1983) was the first to discover that CD9 mAb is a powerful platelet aggregating agent. Subsequent studies of the CD9 function in platelet aggregation have provided much information on the mechanisms by which CD9 mAb induces platelet activation. In fact, since platelet activation is a well defined phenomenon and several parameters of the activation process are readily measurable, most of the understanding we have today on how CD9 may function comes from such experiments.

In their initial report, Boucheix et al. (1983) observed that mAb ALB6 added to platelet-rich plasma (PRP) led to a reproducibly strong platelet aggregation reaction which was preceded by a lag phase. The aggregation was considered specific because no platelet lysis occurred during the assay, as was demonstrated by the absence of LDH release, and because the aggregation could be inhibited by various substances which could not inhibit an immunological agglutination. Furthermore, bivalent mAb seemed to be necessary to bring about an aggregation (i.e. Fab fragments of the CD9 antibody did

not induce the effect). The ability of CD9 mAbs to aggregate platelets was later reported by Gorman et al (1985) using mAbs FMC 8, FMC 48 and FMC 56, by Higashihara et al. (1985) using mAb TP82, by Miller et al. (1986) using mAb AG1, by Seehafer et al. (1988) using mAb 50H.19, and By Hato et al. (1988) using mAb PMA2. The aggregation reactions caused by these mAbs were not believed to be due to antibody-dependent agglutination since Fab fragments were inhibitory to the aggregation induced by the antibodies (Boucheix et al.1983; and Gorman et al., 1985; Miller et al. 1986; our unpublished result) and the column-purified $F(ab')_2$ fragments of the mAb TP82 were as effective as the intact antibody in causing aggregation (Higashihara et al., 1985a). Moreover, mAb FMC 8 Fab fragments, and FMC 56 $F(ab')_2$ were able to enhance the response of platelets to other agonists (Gorman et al., 1985), although an opposite effect was experienced with the mAb ALB6 Fab fragments (Boucheix et al., 1983), a discrepancy that may be explained by the difference in epitopes recognized by the antibodies. These lines of evidence strongly indicate that the antibody induces the aggregation reaction through specific binding to CD9 antigen, not by non-specific binding to Fc receptor, which was shown to be able to activate the platelet when bound by zymosan (Martin et al., 1978). Furthermore, the antibodies failed to agglutinate formaldehyde-fixed or metabolically inhibited platelets, or Glanzman's thrombasthenic platelets which genetically lack the platelet glycoprotein IIb-IIIa receptor (which is involved in aggregation process by binding to fibrinogen) but contain the CD9 antigen (Boucheix et al., 1983; Gorman et al., 1985; Higashihara et al., 1985a; Hato et al., 1988). In addition, a peculiar pattern in dose-response relationship was found in the aggregation study: 1) the CD9 mAbs are potent aggregating agents whose effects drop rapidly at antibody concentrations below 10 μg/ml; 2) a lag phase usually precedes the single wave of aggregation curve (Boucheix et al., 1983; Gorman et al., 1985; Higashihara et al., 1985; Miller et al., 1986; Seehafer et al., 1988).

The potency of the CD9 mAb-induced aggregation reaction may be explained by the finding that CD9 antigen is, besides the von Willebrand factor receptor GPIb-IX and the fibrinogen receptor GP IIb-IIIa, one of the three major surface glycoproteins of platelets (Seehafer et al., 1988). A similar finding was reported by Zola et al. (1984), who observed that CD9 is the major iodinated component of both platelets and NALM-6 cells. Moreover, quantitation of ^{125}I-AG-1 Fab binding to platelets revealed approximately 65,000 binding sites for CD9 mAb's on each platelet (Miller et al., 1986), quite a dense distribution for the size of the platelet. This estimation was also confirmed recently by Hato et al. (1988), who used intact ^{125}I-PMA2 to detect the binding sites and found about 46,000 binding sites per platelet, which is similar to the estimated number of fibrinogen sites (35-45,000) (Shattil, and Brass 1985). This suggests that a 1:1 relationship might exist between them.

G. MECHANISM OF ACTION IN PLATELET AGGREGATION

Although CD9 antigen is clearly involved in a variety of basic cellular functions such as cell activation, proliferation, and cell-cell, or cell-environment interaction, a fundamental question still remains to be answered: how important is CD9 in such cellular activities, and how does CD9 modulate such activities? Since the significance of CD9 antigen in basic cell physiology is only beginning to be recognized, the mechanism by which CD9 functions is poorly understood at the moment. On the other hand, several studies have been carried out to investigate the role played by CD9 in platelet activation. The results from these investigations have shed some light on how CD9 may function in platelets. Although the functions served by CD9 in platelet activation may be quite different from that in other cells, because platelets are very specialized and unique cells, the information obtained is very useful in reaching a full understanding of CD9 function in general.

Since platelet activation is a complex process that can be influenced by a variety of stimuli and involves the interplay of many different mediators, a thorough analysis of the interaction between the CD9 protein and the many platelet agonists would be necessary before any meaningful conclusion can be reached. Various experiments have thus been designed to study the influence of such agonists on CD9-mediated platelet activation. These studies were also aimed to determine if CD9 transduced its signal through any of the known activation pathways utilized by those agonists. The results have established that i) CD9 may activate platelets in a prostaglandin-independent manner, since pretreatment of the platelets with aspirin did not inhibit the CD9 antibody induced aggregation (Gorman et al., 1985; Higashihara et al., 1985), ii) ADP is not likely to be involved in mediating the CD9 signal, since mAb TP82 induced activation was only partially affected at concentrations of apyrase that inhibited ADP induced aggregation, (Higashihara et al., 1985), iii) CD9 does not seem to activate platelets through the thrombin pathway either, since two thrombin inhibitors failed to block the antibody-mediated effects (Higashihara et al., 1985). Furthermore, the mAb induced aggregation has been shown to differ from that involving thrombin by the presence of a distinct lag phase, a slower release of granule, and the lack of requirement for an external calcium source (Rendu et al., 1987).

Although CD9 may be regarded as a unique platelet activating molecule that may not rely on other platelet agonists for its function, the biochemical changes occurring after binding of agonists to the platelet membrane receptors on the other hand, could be useful for understanding how CD9 may carry out its function. Changes in the level of cyclic nucleotides, the influx of calcium, the hydrolysis of membrane phospholipids, and the phosphorylation of critical intracellular proteins are all recognized pathways through which platelet activation is regulated.

1. *Regulation of Calcium Flux*

An increase in cytosolic calcium ion concentration is presently considered to be the trigger for platelet activation, bringing about morphological change, secretion and aggregation. The elevation of intracellular [Ca^{++}] may occur by influx through the plasma membrane or by the intracellular liberation by the stimulation of phosphoinositide metabolism of calcium sequestered in the dense tubular system. The importance of calcium in platelet activation is best exemplified by the ability of an ionophore such as A23187 to activate platelets in the absence of other agonists. This particular reagent is calcium-specific, and has a hydrophobic periphery enabling it to traverse a lipid bilayer in order to allow the passage of extracellular calcium into the cytoplasm.

Although studies of CD9 mAb and calcium channel blockers in fibrin clot retraction had indicated a possible role for CD9 in regulating intracellular calcium, the initial observations that implicated CD9 in controlling calcium flux were, once again, obtained from experiments carried out on platelets. While it was observed that mAb ALB6 caused platelets to aggregate in a specific manner, the aggregation reaction was greatly inhibited by EDTA (a calcium chelator), and by anti-calcium drugs. Moreover, mAb ALB6 Fab fragments were also able to block the activation of platelets by calcium ionophores (Boucheix et al., 1983). Since the antibody did not penetrate into the platelet, the authors suggested that CD9 may either be part of, or control a calcium channel located on the plasma membrane. This inhibitory effect resulting from the removal of external calcium on CD9 mAb induced platelet activation was later confirmed by Higashihara et al. (1985a) using mAb TP82. Furthermore, it was reported that ALB6 mAb was also able to increase the incorporation of $^{45}CaCl_2$ into isolated platelet membrane vesicles enriched in internal membranes, suggesting that the calcium flux was mediated by a local event which was independent of the cytoplasm. The suggestion that CD9 may be a calcium-channel regulator was appealing, since it accounted for the

observations made on clot retraction by fibroblasts during which the inhibition of the retractile activity by calcium-channel blockers was reversed by CD9 mAb. However, although a calcium channel has been described in platelets, it is located in the dense tubular membrane, not the plasma membrane. Furthermore, it has been shown that, in the presence of EDTA, CD9 mAb AG-1 induced platelet shape change (as detected by aggregometry and scanning electron microscopy) without provoking an appreciable aggregation reaction (Miller et al. 1986). In addition, the presence of calcium chelating agents EDTA and EGTA uncoupled the granule release reaction from the aggregation reaction (Higashihara et al., 1985b; Rendu et al., 1987). Since both the morphological change and granule release clearly require the participation of calcium ions, these observations indicate that the intracellular calcium must come from internal storage sites, the liberation of which is regulated by other molecules generated or activated as a result of antibody binding to CD9. This conclusion is well supported by evidence for CD9 mAb-induced calcium release in intact tumor cells (see Section F4). The increase in intracellular [Ca^{++}] induced by cross-linking the bound mAb 50H.19 on the surface of NALM-6 cells was almost completely eliminated by prior treatment with calcium channel blockers, while the removal of external calcium by addition of EDTA only halved the increase (Yip and Shaw, 1988). Since the CD9-mediated signal is able to mobilize the internal calcium pool, the inhibition of antibody-induced platelet aggregation by calcium channel blockers is likely due to the blockage of both the external flux and the internal release by the blockers. Indeed, when extensively washed platelets were suspended in Ca^{++}-free media (without added calcium blockers), the reduction in the antibody-induced aggregation could no longer be observed (Yip and Shaw, 1988). These experiments therefore suggest that although CD9 seems to be involved in the regulation of intracellular calcium levels, it may only do so indirectly by generating mediators.

2. *Generation of Diacylglycerol and Inositol Trisphosphate*

Recent evidence has shown that the phosphoinositide cascade, like the adenylate cyclase cascade, converts extracellular signals into intracellular ones, and hence evokes a wide variety of responses in many kinds of cells (Berridge, 1984, 1987; Hokin, 1985). The intracellular messengers formed by activation of this pathway arise from phosphatidylinositol 4,5-bisphosphate (PIP_2), a phospholipid in the plasma membrane. The binding of an agonist to a cell surface receptor leads to the activation of a membrane bound phospholipase C (PLC) (probably through G-protein) that hydrolyzes PIP_2, generating diacylglycerol (DAG) and inositol trisphosphate (IP_3). While IP_3 causes the rapid release of Ca^{++} from intracellular stores (Berridge, 1987), DAG activates protein kinase C (PKC) (Bell, 1986), an enzyme that phosphorylates serine and threonine residues in many target proteins. Both the increase of intracellular calcium and the activation of PKC are known to correlate with cellular stimulation. Since the activity of PKC is dependent of the presence of calcium, most effects of IP_3 and DAG are synergistic.

Along with the generation of hydroxy peroxide and TXA_2 through the release of arachidonic acid from the membrane phospholipids by phospholipase A_2, the phosphoinositide cascade has recently been shown to be an important alternative pathway in platelet activation. Phosphoinositide breakdown followed by DAG formation is known to be involved in platelet activation by thrombin, collagen and platelet activation factor 4 (Rendu et al., 1983; Handin, 1987). IP_3 mediates the movement of calcium into the platelet cytosol and stimulates, through myosin light chain kinase, the phosphorylation of myosin light chain (p20). The latter interacts with actin to facilitate granule movement and platelet shape change (Daniel et al., 1981). DAG activates protein kinase C which, in turn, phosphorylates the IP_3 phosphomonoesterase (p47, also

referred to as p40) involved in terminating IP_3 mediated calcium flux (Connolly et al., 1986).

The studies of CD9 induced calcium flux clearly demonstrate the existence of molecules acting as intermediate messengers to regulate calcium release from internal storage pools. The lag phase in CD9 mAb-induced platelet aggregation also suggests this possibility. Since cyclooxygenase inhibitors can block platelet activation to varying degrees following antibody binding, the CD9-induced signal seems to depend in part on the breakdown of arachidonic acid from platelet phospholipids and subsequent formation of TXA_2. The TXA_2 receptor is in turn an agonist of phospholipase C which hydrolyses membrane phospholipids. CD9 does not possess a glycophospholipid anchor which might be susceptible to the enzyme since it cannot incorporate radiolabeled ethanolamine, or myo-inositol (Seehafer et al., unpublished).

That CD9 mAb binding to platelets indeed resulted in DAG production was demonstrated by Higashihara et al. (1985b). By pre-loading the platelets with the [^{14}C] serotonin (to label the contents of dense granules) and by using a radioimmunoassay to detect platelet factor 4, β-thromboglobulin (from alpha granules) and N-acetyl-β-D-glucosaminidase (an endoglycosidase from lysosomes), these investigators observed a CD9 mAb TP82-induced release of platelet granules in the absence of aggregation. The release phenomenon proceeded in a dose-dependent manner for both the plasma rich platelets and the washed platelets, suggesting that mAb TP82 induced release was essentially independent of aggregation. More importantly, a rapid and transient production of DAG accompanied by phosphorylation of the p20 and the p47 proteins occurred prior to the release of granule and peaked at the onset of aggregation. Taken together, the data strongly indicated that phosphoinositide breakdown might play an essential role in CD9 mAb-induced platelet activation. This notion is further strengthened by the recent findings of Rendu et al. (1987), who reported that mAb ALB6 induced the

breakdown of PIP_2 with a concomitant synthesis of phosphatidic acid and the phosphorylation of the p20 and p47 proteins. More significantly, these processes occurred in the absence of external calcium, suggesting that the activation of the PIP_2 cascade by mAb binding is not secondary to the changes in intracellular calcium. Finally, it is interesting to note that protein kinase C is involved in the phosphoinositide cascade and a serine and threonine specific kinase is present in the CD9 mAb immunocomplexes discussed earlier (see Section E4). It is tempting to speculate that the kinase activity coprecipitating with the CD9 antigen may represent the same protein kinase C.

CD9 may therefore transduce its activating signal(s) through the phosphoinositide cascade. Such a process is consonant with the calcium flux and the protein phosphorylation events observed during the activation reaction. However, the mechanism(s) whereby CD9 activates phospholipase C is, at present, poorly understood. Since CD9 is an external membrane protein without a transmembrane domain, and PLC is located in the inner part of the membrane, it is unlikely that a direct interaction exists between the two proteins. At least one other molecule should be involved in transducing the signal from CD9 to PLC. Since GTP-binding proteins (G proteins) are believed to relay excitation signal(s) from activated receptors to PLC (Gilman, 1987), (as evidenced by the stimulation of the PIP_2 breakdown with hydrolysis-resistant analogs of GTP and by the inhibition of such breakdown with G protein inhibitor pertussis toxin), it is possible that G protein(s) may serve as such relaying molecules. This, too, could partially explain the lag phase experienced in platelet aggregation by CD9 mAbs.

3. *Interaction with Other Membrane Glycoproteins*

The platelet membrane is the focus of molecular interactions involved in platelet activation. The platelet membrane glycoprotein complex, Ib-IX (GP Ib-IX), serves as the receptor for von Willebrand factor (vWF), and is involved in primary hemostasis by

binding to vWF deposited on the collagen fibrils of the exposed subendothelium which enables platelets to adhere to the vascular subendothelium. GP Ib-IX also provides attachment sites for platelet actin filaments through actin binding protein. The membrane calcium-dependent heterodimer glycoproteins IIb and IIIa (GP IIb-IIIa) form a complex which is involved in platelet aggregation. The complex undergoes a conformational change upon platelet activation and serves to bind fibrinogen which participates in linking adjacent platelets together to form aggregates. Clot retraction by platelets is also dependent on GP IIb-IIIa, since the cytosolic extension of the protein dimer provides the attachment for platelet actomyosin (Phillips, 1980, 1984; Berndt and Caen, 1984; Fox et al., 1988; Phillips et al., 1988). In addition, platelet aggregation that occurs at high shear rates appears to require an interaction between the larger von Willebrand factor multimers and both GP Ib-IX and GP IIb-IIIa (Peterson et al., 1987). In contrast to these membrane glycoproteins, relatively little is known concerning the receptors for various agonists that regulate platelet function and the signal transducing elements for the membrane. Studies using radiolabeled ligands have established that the platelet plasma membrane has specific receptors for a number of platelet-stimulating agents, including thrombin, adenosine diphosphate (ADP), serotonin, thromboxane, and prostaglandin. However, the membrane proteins that serve as specific and functional receptors for these agents have not been conclusively identified.

The binding of platelet GP IIb-IIIa complex by fibrinogen is an obligate step in platelet aggregation (Bennett and Vilaire, 1979). Besides serving to bring together adjacent platelets to form aggregates, the GP IIb-IIIa complex is also involved in further activation of the platelets, perhaps by a positive feedback activation process. This was demonstrated by the aggregation of platelets with a monoclonal antibody directed against the 23 kDa β-chain of the GP IIb component (Jennings et al., 1985). Both the intact immunoglobulin as well as the Fab fragments activated the platelet in a calcium-

dependent manner, indicating that the GP IIb or in this case the fibrinogen receptor also delivers an activation signal. It is also known that the fibrinogen receptor/GP IIb-IIIa complex is exposed only after platelets have been activated to some extent by various agonists such as ADP, epinephrine, thrombin, collagen, and prostaglandin endoperoxidase, which provide signals necessary for exposure of the fibrinogen binding site in a calcium-dependent processes (Bennet et al., 1985; Shattil et al., 1985). Little is known, however, about the mechanism by which this is achieved. Conformational changes following agonist binding to platelets have been proposed to be required for receptor exposure (Shattil et al., 1985).

Several lines of evidence have implicated CD9 in modulating the interaction between fibrinogen and its receptor. CD9 mAb ALB_6 Fab fragments were shown to inhibit aggregation induced by ADP, collagen, thrombin, and even ionophore A 23187, suggesting that the Fab fragments interfere with a mechanism common for all aggregation pathways (Boucheix et al., 1983). Furthermore, the aggregation or secretion responses to CD9 mAbs were abolished with thrombasthenic platelets deficient in GP IIb-IIIa complexes, but possessing CD9 antigen. A similar observation was made in the presence of an anti-GP IIb-IIIa monoclonal antibody, suggesting that the CD9 antibody-induced platelet aggregation was mediated by fibrinogen binding to the GP IIb-IIIa complex (Boucheix et al., 1983; Higashihara et al., 1985a; Gorman et al., 1985; Miller et al.; 1986). Pre-incubation of the platelets with the inhibitory ALB6 Fab fragments greatly reduced binding of the ^{125}I fibrinogen to platelets activated by ADP, indicating that the Fab fragments blocked the exposure of the platelet fibrinogen binding sites (Boucheix et al., 1983). In addition, although extracellular fibrinogen was not required for TP82-induced aggregation, it shortened the time required to reach half-maximal aggregation (Higashihara et al. 1985a). This potentiating effect by fibrinogen on the CD9 mAb-induced aggregation reaction was also noted with mAb AG-1 (Miller et al.,

1986). Interestingly, after platelets were separated from serum by Sepharose 2B chromatography (instead of repeated washing and centrifugation as in other aggregation studies), they were no longer responsive to CD9 mAb activation. Exogenously added fibrinogen however, was able to restore the aggregation capability of such platelets by mAb 50H.19 (our data). Finally, mAb TP82 produced a transient decrease in light transmission accompanied by an incomplete aggregation reaction in aggregometry assays using thrombasthenic platelets (Higashihara et al., 1985a), demonstrating that CD9 mAb-induced platelet activation rely on the formation of GP IIb-IIIa complexes in a dose-dependent manner.

Although the mechanism by which CD9 modulates the binding of fibrinogen to its receptor is unknown, two proposals can be made to explain how this modulation event is accomplished. On the one hand, CD9 may associate directly with the receptor and strengthen the receptor's binding to fibrinogen by inducing a structural change. Indeed, exposure of the GP IIb-IIIa complex is believed to involve conformational changes of the glycoproteins. It was recently observed that CD9 antigen could be cross-linked to the GP IIb-IIIa complex upon platelet activation by monoclonal antibody (Slupsky et al. submitted), indicating that physical contact between the two molecules may indeed exist, at least during platelet activation. Surprisingly, CD9 mAbs FMC 56, ALB6 and BU-16 were also reported to induce vWF binding to platelets (Gralnick et al., 1987). Furthermore, it was even suggested that CD9 may be physically associated with the GP Ib-IX complex, since the GP Ib glycoprotein was found to coprecipitate with CD9 antigen during immunoprecipitation with mAb AG-1(Miller et al., 1986). Considering the dense distribution of CD9 on the platelet surface, its long chain fatty acid ligands and its unique attachment to the plasma membrane, it is possible that CD9 may serve to facilitate the binding of both the GP Ib-IX and the GP IIb-IIIa complex to their ligands. It is equally possible that an even bigger complex could be formed among the three

molecules. Alternatively, CD9 may modulate the GP IIb-IIIa complex only indirectly through the activation of various cascades. This is supported by the ability of many platelet agonists, including ADP, epinephrine, thrombin, collagen, arachidonate, prostaglandin endoperoxide analogues, and platelet activating factor, to induce fibrinogen binding to platelets. All of these are released from the platelet granules during the activation process triggered by CD9 mAbs. Furthermore, in the absence of the GP IIb-IIIa complexes, as in the case of thrombasthenic platelets, CD9 mAb PMA2 was still capable of inducing ATP and thromboxane B2 secretion (Hato et al., 1988), suggesting the CD9 mediated signal is independent of GP IIb-IIIa complex formation. Similar suggestions arise from the shape changes induced by CD9 mAb in thrombasthenic platelets (discussed earlier in the same Section). A more convincing argument may be taken from the recent demonstration that combined treatment of platelets with both aspirin (an inhibitor of thromboxane synthesis) and creatine phosphate/creatine phosphokinase (an ADP scavenger) abolished fibrinogen binding and aggregation induced by CD9 mAb PMA2 (Hato et al., 1988), although treatment with either reagent did not affect the activation process. This indicates that CD9 may expose fibrinogen receptors through both ADP and thromboxane, and that either one or both may expose the receptors to bring about full platelet activation. Finally, indirect exposure of the GP IIb-IIIa complex by CD9 is also supported by the finding that CD9 mAbs were able to induce the binding of both fibrinogen and vWF to platelet (Gralnick et al., 1987). Conceivably, therefore, the binding of these ligands to their respective receptors is under the influence of mediators elaborated during platelet activation.

Whether or not CD9 modulates the binding of agonists to their membrane receptors, indirectly or directly, it is important to point out that the two possibilities are not mutually exclusive. In fact, it is possible that while CD9 becomes associated with the GP IIb-IIIa complex (and/or GP Ib-IX) to facilitate ligand binding, it may also transduce

the signal(s) generated as a result of receptor occupancy. The signal transduction events may then generate a variety of mediators which could further activate the receptors. This process eventually may lead to a full activation of the platelet aggregation process.

4. *A Unifying Model for CD9 Function in Platelet Activation*

It is clear from the previous discussion that the binding of anti-CD9 mAbs does induce platelets to aggregate, however, it is still not quite clear how. The following model is necessarily more speculative, than explicative, and is intended primarily as a framework to connect the diverse observations of CD9, and platelet function. CD9 appears to activate the phosphoinositide cascade, and thereby produce the mediators inositol trisphosphate and diacylglycerol. It may also enhance platelet aggregation by promoting the binding of fibrinogen and possibly von Willebrand factor to their receptors. The mechanism of receptor priming is not clearly understood, but appears to involve both conformational changes, and intracellular signals including changes in intracellular Ca^{++}. CD9 binding to adhesion protein receptor(s) may produce conformational changes enhancing their affinity for their ligands, and also transduce signal(s) from the receptor-ligand complex to other proteins, and possibly phospholipase C via G-proteins. This would lead to production of IP_3 and DAG, and therefore to the activation of protein kinase C. PKC would in turn phosphorylate key proteins involved in platelet aggregation. These events could be amplified by other agonists released by the partly-activated platelets. The platelet activation reaction would thus represent the end point of many stimuli acting synergistically through different pathways. CD9 mAb binding to CD9 antigen may therefore mimic a conformational change originating from a ligand-receptor interaction, short-circuiting the otherwise physiological initiation signal, and setting in motion a chain of events resulting in platelet activation. Figure 1 is a simplified graphic depiction of the proposed model by which CD9 participates in platelet activation.

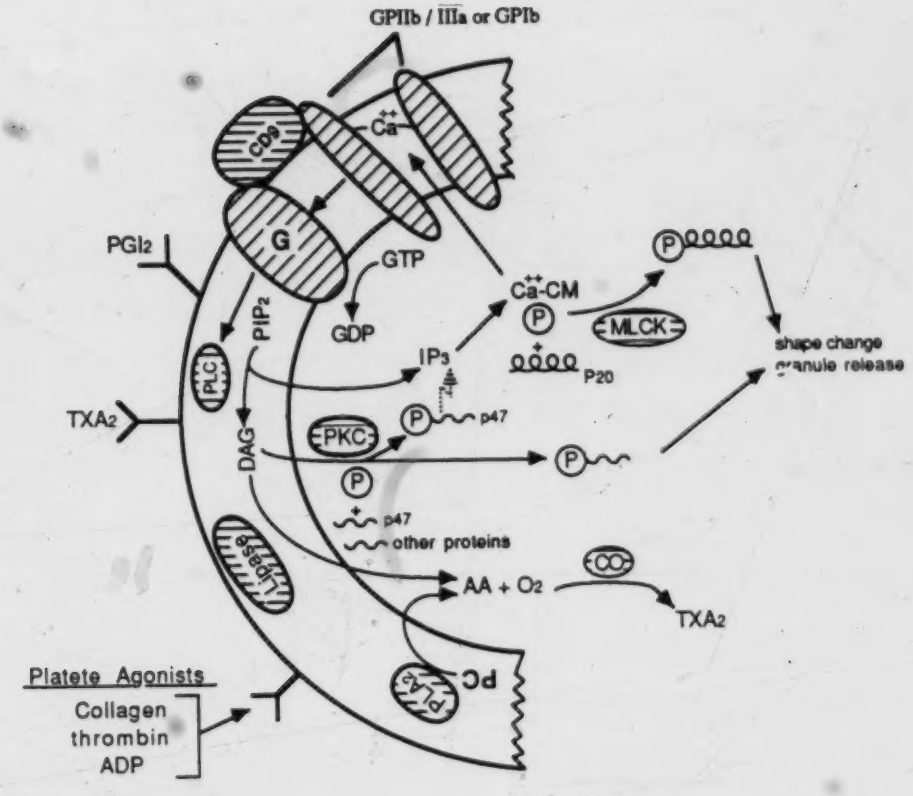


Figure 1. Model of CD9 function in platelet activation. Binding of agonists such as thrombin, epinephrine, or collagen initiate the hydrolysis of membrane phospholipids, mobilization of intracellular calcium, and phosphorylation of critical intracellular proteins resulting in shape change, generation of intracellular mediators, and granule release. CD9 may increase the binding affinity of adhesion protein receptors such as GP IIb/IIIa and GP Ib, and may additionally transduce the signals from the occupation of these receptors by their ligands.

Abbreviations: G, GTP binding protein; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLA_2, phospholipase A_2; PC, phosphatidylcholine; AA, arachidonic acid; CO, cyclooxygenase; O_2, oxygen; TxA_2, thromboxane A_2; PKC, protein kinase C; DAG, diacylglycerol; PLC, phospholipase C; PGI_2, prostaglandin I_2; IP_3, inositol trisphosphate; p47, IP_3 phosphomonoesterase; MLCK, myosin light chain kinase; p20, myosin light chain; GPIIb/IIIa, fibrinogen receptor; GPIb, Von Willebrand factor receptor; Ca^{++}-CM; calcium-calmodulin.

The involvement of CD9 in the process by which platelet receptors bind their ligands is very significant, since some of these receptors belong to the integrin superfamily (see Section F4). The GP IIb-IIIa receptor complex, for example, is one of the first integrin molecules described (Hynes, 1987). In addition to fibrinogen, the integrins also bind to several other adhesion molecules such as fibronectin, von Willebrand factor, and vitronectin by recognizing the RGD sequence carried by these ligands (Parise and Phillips, 1986; DeMarco et al., 1986; Pytela et al., 1986). The receptor complex may also bind matrix proteins such as thrombospondin and collagen, each of which also contain the RGD sequence (Plow et al., 1985). Furthermore, molecules serologically related to the GP IIb-IIIa complex have been identified in a wide range of cell types such as fibroblasts, leukocytes, smooth muscle, and endothelial cells (Phillips et al., 1988). Very late antigen (VLA), the integrin initially found on activated T cells, has also recently been found on platelets. Platelet GP Ia-IIa (VLA-2) serves as the receptor for collagen, while GP Ic-IIa (VLA 5) binds to fibronectin. Recently, the human fibroblast class II extracellular matrix receptor was shown to be identical to the platelet GP Ia-IIa complex (Kunichi et al., 1988). It is therefore becoming more and more apparent that most adhesion receptors on platelets have similar, if not identical, counterparts on a variety of other cell types whose function depends on cell-cell or cell-environment interaction. In turn, platelet aggregation should now be considered within a broader context of cellular adhesion. It is not without reason to conclude from the results of platelet aggregation studies that the overall function of the CD9 antigen in nucleated cells may be to facilitate adhesion receptors to bind their ligands and to transduce the signal(s) generated from the ligand-receptor complexes.

H. CLINICAL APPLICATION OF CD9 mAbs

The availability of monoclonal antibodies directed against human tumor antigens has opened the way to new immunodiagnostic and immunotherapeutic applications which are having a major impact in cancer diagnosis, treatment, and prognosis. Microscopic, as well as macroscopic tumors undetectable by conventional imaging techniques, have been rendered detectable by immunodiagnostic imaging methods employing the radiolabeled monoclonal antibodies as imaging materials. Classification and staging of some poorly differentiated cancers have been facilitated by the use of monoclonal antibodies. Cytotoxic monoclonal antibodies have also been successfully used to destroy antigen-bearing tumor cells both *in vitro* and *in vivo*. Cytotoxic agents such as radioisotopes, anti-metabolites and toxins, microspheres, and metal colloids, have been chemically linked to monoclonal antibodies to create a new generation of immunotherapeutic agent, the so called "magic bullets". The latter are currently being evaluated for their clinical use in separating tumor cells from the normal cells, and in treating certain malignancies. Monoclonal antibodies against serum tumor markers such as carcinoembryonic antigen and alphafetoprotein have been used successfully in monitoring the treatment and relapse of some cancers as well (Shiku, 1987; Fodstad, 1988; Maners, 1988; Ricehmann, 1988; Kalofonos, 1988).

1. *CD9 mAbs in Cancer Diagnosis*

Since CD9 is strongly expressed by a variety of solid tumors, as well as in tumors that are derived from antigen-negative progenitor tissues, several studies were conducted to determine if CD9 mAb could be used as an imaging material in detecting early metastatic antigen-bearing solid tumors. Radio-labeled mAb 50H.19, for example, was injected *in vivo* to detect metastatic breast cancer (Maclean et al., personal communication). However, the results from these studies were disappointing, mainly because of high background signals which compromised the interpretation of the

images. Considering the wide distribution of CD9 antigen in normal tissues such as epithelium, endothelium, macrophage and mucosal membrane, a low signal to noise ratio was anticipated.

In contrast to the ubiquitous expression of CD9 antigen in non-hematopoietic tissues, the presence of the antigen is restricted in hematopoietic cells, and therefore may be a useful phenotypic marker of leukemias. Null ALL cells are currently divided into four subtypes on the basis of reactivity with several CD monoclonal antibodies, including the CD9 mAb (Jones et al., 1982). Studies are being conducted to determine if any prognostic significance can be applied to these ALL subgroups, and to determine whether there is a clinical relevance to the presence or absence of the CD9 antigen on cells of patients with AML. In addition, the CD9 antigen was reported detectable in the plasma of c-ALL patients. Furthermore, the plasma antigen was only present in patients at the time of diagnosis and during treatment and became undetectable when the patients had achieved a complete remission (Komada et al., 1983). It is therefore possible to use the plasma antigen to monitor the tumor burden. However, since the antigen is probably not secreted by the tumor cells (see Section E3), further studies are required to evaluate this possibility.

2. *CD9 mAbs in Bone Marrow Purging*

Therapy for human leukemia, lymphoma and solid tumors is often limited by the toxicity of chemotherapy and radiotherapy to normal hematopoietic stem cells. For hematologic malignancies, particularly acute leukemia (but also for chronic myelogenous leukemia, malignant lymphoma, and myeloma), supralethal chemoradiotherapy has been given in combination with bone marrow transplantation (BMT) to reconstitute marrow function. Due to the difficulty in obtaining HLA-identical donors, genotypically nonidentical donors have been used as marrow sources. Unfortunately, this carries the risk of acute and/or chronic graft-versus-host disease, in addition to graft rejection. An

alternative approach to prevent the eradication of bone marrow function by chemoradiotherapy has been to remove and store part of the patient's marrow prior to therapy, followed by reinfusion of the stored marrow as an autologous bone marrow transplant. The major concern is the likelihood that the patient's bone marrow will be contaminated by residual malignant cells, even during complete remission. Several techniques for removal of these residual leukemic cells have been investigated. Most of the efforts, however, have centered on the use of monoclonal antibodies capable of selectively reacting with and removing residual leukemic cells from the "remission" autologous bone marrow. The process whereby this is achieved is termed bone marrow purging.

Although the universal presence of CD9 antigen on normal tissues places a major limitation on the *in vivo* use of CD9 mAb as an immunotherapeutic agent, the specificity of the antibody to ALL (LeBien et al., 1981, 1982) and ANLL (Ashman et al., 1987) makes it an ideal candidate for use in purging leukemic bone marrow. However, because CD9 is also expressed on some hemopoietic progenitor cells, it was important to determine if removal of these CD9 positive cells would affect host marrow reconstitution by the treated marrow sample. Using mAb DU-ALL-1 induced complement-mediated cytotoxicity in an *in vitro* colony forming assay, Levine et al. (1981) had shown that CD9 was not present on the progenitors of granulocytes and macrophages. A similar study using mAb BA-2 established the absence of the antigen on pluripotential and committed hematopoietic progenitors (Ash et al., 1982; LeBien et al., 1986). Although the antigen was present on megakaryocytes, neither myeloid nor erythroid colony formation from marrow stem cells were affected by the treatment with mAb CALL1 (Deng et al., 1983; Bradstock et al., 1986). Stepan et al. (1984) first reported the use of CD9 mAb for the destruction of leukemic cells. mAb BA-2 caused an efficient lysis of the ALL cell lines HPB-NULL and REH through complement mediated cytotoxicity as

assayed by dye exclusion and ^{51}Cr release. The ability of CD9 mAb-induced complement-mediated cytolysis in cALL cells was confirmed by an *in vitro* plating assay using mAb FMC-8 (Bradstock et al., 1986). A more efficient and selective elimination of leukemic cells was achieved by combining three mAbs, BA-1, BA-2, and BA-3, directed against different antigens present on the leukemic cells. No adverse effect on the pluripotent normal stem cells was observed as a result of such treatment (LeBien et al., 1986). The combination was capable of lysing more than 95% of target cells in a ^{51}Cr release assay, even in the presence of excess marrow cells. The increased lysis was probably due to the higher number of membrane attack units formed on each cell, instead of a higher antibody/cell ratio, since combination of several CD9 mAbs did not result in an increase in complement-mediated cytotoxicity (Slaper-Cortenbach et al., 1987). These studies clearly suggest the potential use of CD9 mAbs in purging autologous bone marrow transplants in acute leukemic patients. Its usefulness in clinical application, however, still awaits further clinical trials.

I. THESIS OBJECTIVES

The association of the lymphocyte differentiation antigen CD9 with a wide variety of excitable, secretory, and proliferating tissues strongly supports the involvement of CD9 in the regulation of a fundamental cellular function. The biological functions displayed by the antigen in lymphocyte activation, in fibroblast clot retraction and in platelet aggregation as well as in the modulation of calcium flux and phosphoinositide breakdown, suggest that while it may interact with some other plasma membrane receptors to promote cell-environment interaction, it may also participate in the transduction of membrane-mediated signals. Its unique mode of attaching to the plasma membrane and its tissue-specific glycosylation profile qualify the antigen as a novel cell surface glycoprotein.

To further study the biochemical structure and biological function of the CD9 antigen and to explore the molecular mechanisms by which CD9 exerts its function, we deemed it necessary to obtain the gene encoding the protein. The amino acid sequence predicted from the DNA sequence would then help to determine both the primary and the secondary/tertiary structure of the protein as well as its relationship with other known proteins, especially the membrane receptors involved in cell-environment interaction. Furthermore, monoclonal antibodies generated against the exposed regions of the protein could serve as probes to study the functional domains of the molecule, and thus determine how CD9 interacts with other proteins. The gene cloning would enable one to answer the question regarding whether the different components of the CD9 antigen are derived from the same or different genes. Moreover, the cDNA probe obtained could be used to study the control of CD9 gene expression in different tissues and during cell activation and differentiation. The cDNA probe could also be used to isolate CD9 mRNA from a variety of human tissues, and from other species so that the diversity, and evolutionary relationships of CD9 could be compared. Finally, isolating the CD9 gene would make possible the study of the role of CD9 in cell proliferation by co-transfecting recipient cells with CD9, and oncogenes.

The primary aim of this study was to accomplish the cloning of the CD9 cDNA. Since no amino acid sequence information was available, it was first necessary to purify a large amount of antigen from CD9 bearing cells through mAb affinity chromatography. The purified antigen would next be used to raise polyclonal antibody against the CD9 antigen, which in turn would be utilized for isolating the CD9 cDNA from a λgt11 cDNA expression library constructed from the antigen producing tumor cell line. The purified antigen would also be used to generate proteolytic peptide fragments from the CD9 antigen in an attempt to perform peptide micro-sequencing.

CHAPTER II

MATERIALS AND METHODS

A. CELL CULTURE AND ANIMALS

1. *Culture Medium*

Cultured cells were routinely maintained in RPMI 1640 growth medium (Gibco, Burlington, Ontario) supplemented with 20 mM sodium bicarbonate, 100 I.U./ml penicillin G, 100 μg/ml streptomycin sulphate, and 10% heat inactivated (56°C for 30 min) fetal calf serum (FCS) (Gibco). Growth medium with a shelf life in excess of one month was replenished with 1% (w/v) L-glutamine. For convenience, growth medium supplemented with 10% or 20% FCS will be referred to hereafter as 10% RPMI and 20% RPMI respectively. Stocks of serum-free growth medium were sterilized by filtration through a 0.2 μm Millipore filter unit and stored at 4°C until used. Cell cultures were grown in disposable plastic tissue culture flasks (Corning) and maintained at 37°C in a humidified atmosphere (70% humidity) with 5% CO_2 in air.

2. *Cell Lines*

The human cell lines NALM-6 (ALL) and SKOSC (osteogenic sarcoma) were obtained from Drs. Theodore Zipf, University of Calgary (Calgary) and Jorgen Fogh, Memorial Sloan-Kettering Cancer Center (New York) respectively. The leukemic cell line Wacko was established in our laboratory from the spleen of a hairy cell leukemia patient. The origin and establishment of the human colorectal tumor cell line SW480 has been described elsewhere (Maclean et al., 1982). The human choriocarcinoma cell line JEG and the Burkitt lymphoma cell line Raji have been passaged in our laboratory for many years. The human T-cell leukemia line Jurkat and the SV40-transformed simian fibroblast cell line COS-7 were kindly provided by Dr. Verner Paetkau, University of Alberta (Edmonton). The human hepatoma cell line HepG2 was donated by Dr. Barbara

B. Knowles (The Wistar Institute, Philadelphia, PA) and the human megakaryocyte cell line HEL was supplied by Dr. Patrice Mannoni (Institut J. Paoli-I. Calmette, Marseilles, France). The mouse immortalized fibroblast cell line NIH 3T3 cells as well as NIH 3T3 cells stably transfected with the plasmid expression vector pSV2neo and with the recombinant vector pEJneo [pSV2neo carrying the human c-ras^{Ha} oncogene (activated by a mutation at the 12th codon of the first exon)] were obtained from Dr. Remy Aubin in the laboratory of Dr. Malcom Paterson, University of Alberta (Edmonton).

3. *Cell Culture Method*

All cell lines were routinely cultured in RPMI medium, whereas NIH 3T3 cells were maintained in the high glucose Dulbeco's Modified Eagles Medium (DMEM) supplemented with the additives described above. Cells growing in suspension were seeded at a density of 1-2 x 10^4 cells/ml and were harvested at a maximum density of 2 x 10^6 cells/ml unless otherwise stated. Cells growing as monolayers were passaged at ratios of 1/4 to 1/10 (depending on the cell line) immediately upon reaching confluency using 25 mM EDTA and 0.05% trypsin to disperse the monolayers. Cell lines were also frozen at a density of 1 x 10^6 cells/ml in 20% RPMI containing 10% dimethylsulfoxide (DMSO) (Fisher Scientific) in 2 ml pro-vials (Cooke Laboratories, Alexandria, Virginia). Vials were placed at -70°C overnight in a styrofoam box and then transferred to liquid nitrogen for long term storage. To initiate fresh culture, frozen cell stocks were thawed at room temperature, diluted with an equal volume of FCS, resuspended in 20% RPMI at sub-confluent cell density and passaged into regular medium.

4. *Platelets*

Citrated blood was obtained from healthy volunteers and centrifuged in an IEC Centra - 7R^{TM} clinical centrifuge (International Equipment Company, Needham Heights, MA) at 800 r.p.m. for 10 min at 20°C to obtain platelet-rich plasma. Platelets were pelleted at 3,000 r.p.m. for 10 min and washed three times with PBS before they

were solubilized. Out-dated human platelets were obtained from the Canadian Red Cross Blood Transfusion Center (Edmonton) in sealed plastic bags (50 ml per bag) and were kept at 4°C for 1 to 2 weeks before being utilized.

5. *Activated T-cells*

Human peripheral blood mononuclear cells were isolated from heparinized whole blood samples obtained from healthy donors. Platelet rich plasma was removed by centrifugation as described above. The leukocyte rich interphase layer was then recovered and added to an equal volume of 10% RPMI medium. The mixture was next layered on top of an equal volume of a Ficoll/Hypaque gradient solution, and separated in an IEC Centra - 7R™ centrifuge at 1,500 r.p.m. at room temperature for 10 min. The mononuclear cells were recovered from the interphase layer, washed three times with PBS, and resuspended in 10% RPMI medium. Adherent cells were removed by plastic adherence at 37°C for 60 min. Non-adherent cells in the supernatant was recovered and further purified by affinity chromatography using a Nylon-wool column (Leukopac Senwell Company, Norton Grow, IL). Cells eluted from the column were then treated with 5 mM L-leucine-methyl ester (Sigma) at 22°C for 40 min to remove remaining macrophages in order to obtain a homogeneous population of T cells. The purified T cells were finally washed three times with PBS and resuspended in 10% RPMI. T cells were stimulated with 10 μg/ml or 3 μg/ml of phytohemagglutinin (PHA) and 5 μg/ml phorbol ester (PMA) for 84 hours before they were harvested.

6. *Animals*

Female Balb/c mice (4 to 6 weeks old) were purchased from the Health Sciences Laboratory Animal Services, Faculty of Medicine, University of Alberta, and were maintained in the animal facility of the Department of Radiation Biology, Cross Cancer Institute (Edmonton). Male Flemish Giant, Lop-Ear cross bred rabbits (3 to 4 months old) were raised and maintained by the Bioscience Animal Service, Faculty of Science,

University of Alberta (Edmonton). Immunization of mice was carried out by the author whereas rabbits were immunized by the Bioscience Animal Service.

B. IMMUNOBIOCHEMICAL CHARACTERIZATION OF CD9

1. *Antibodies*

mAb 50H.19 stocks used in this study were obtained from a mouse hybridoma, and were produced as outlined previously (Maclean et al., 1982). The antibodies were partially purified from ascites fluids by ammonium sulfate precipitation as follows; saturated ammonium sulfate (SAS) was prepared by dissolving 90 g ammonium sulfate (Fisher Scientific) in 100 ml boiling H_2O and the solution was filtered through a Whatman No.1 filter paper while still hot. The pH was then adjusted to 7.4 by adding 1 M NH_4OH. Ascites fluid was processed by first removing the fibrin clot with a glass rod and then spinning in a Sorvall SS34 rotor at 3,000 r.p.m. at 4°C for 10 min to remove cells and debris. Thirty-two ml of SAS was then added slowly to every 48 ml of ascites fluid with constant stirring. After allowing to stand at 4°C overnight, the precipitate was centrifuged in a Sorvall SS34 rotor at 10,000 r.p.m. (4°C) for 10 min. The supernatant was discarded and the pellet was suspended in 48 ml of H_2O. Thirty-two ml of SAS was added and the precipitation and centrifugation regimen performed once more. The pellet was finally resuspended in a minimal volume of H_2O and dialyzed extensively against 0.15 M NaCl (Spectrapor membrane tubing, 12,000 to 14,000 molecular weight cut-off). The dialyzed antibody solution was then spun at 10,000 r.p.m. (4°C) for 10 min in a Sorvall SS34 rotor to remove insoluble materials. The protein concentration was estimated by the Lowry assay (Lowry et al., 1951) with bovine serum albumin (BSA) serving as the protein standard. mAb 9H.1 was raised against human CML cells and recognizes human β2 microglobulin both in free form and complexed to HLA-A,B,C heavy chains (Maclean et al., 1982). mAb BA-2 was

purchased from Hybritech (through Boehringer Mannheim, Dorval, Quebec). mAb ALB6 was obtained from the Daymar Laboratory (Toronto, Ontario).

To prepare the cell line absorbed polyclonal antibody against CD9, 10^7 NALM-6, HEL, and Raji cells were first washed with PBS and resuspended in 1 ml of polyclonal antibody diluted in PBS (1/100, the production of the anti-CD9 polyclonal antibody is presented in a later section). The cell suspension was rotated gently at 4°C overnight, after which the cells were pelleted by centrifugation for 2 min in an Eppendorf centrifuge. The supernatant was recovered and the activity of the absorbed polyclonal antibody against CD9 antigen was determined by indirect immunofluorescence microscopy using NALM-6 cells and by Western blotting of the platelet lysate.

2. *Indirect Immunofluorescence Microscopy and Flow Cytometry*

Cells recovered from tissue culture were washed twice with PBS and incubated for 30 min at 4°C with saturating titers of mAb 50H.19 or polyclonal antibodies against CD9 antigen. Following two washes with PBS, the cells were next counterstained for another 30 min at 4°C with fluorescein-labeled $F(ab')_2$ fragment goat anti-mouse or goat anti-rabbit IgG (Fc fragment) (Cappel, Malvern, PA) at 1: 8 dilution. Control cells were exposed to non-reactive as well as reactive antibodies. After washing the samples twice with PBS, the cells were examined under a fluorescence microscope for the presence or absence of fluorescence staining. Alternatively, proportion of cells stained by fluorecence was determined by fluorecence activated cell sorter (FACS-I, Becton Dickinson, Mountain View, CA).

3. *ELISA*

Because of the simplicity and sensitivity of the technique, the enzyme-linked immunosorbent assay (ELISA) was used to titre the polyclonal antibody (Engvall and Perlamnn, 1971). Approximately 10^{10} platelets were washed twice with PBS, pelleted, and resuspended in 10 ml of PBS. The platelets were lysed by repeated freeze-thaw

cycles (-70°C and 37°C), followed by sonication (Ultra-Turrax T18, Terochem Laboratory Ltd. Edmonton, Alberta). The cell debris was removed by centrifugation in Sorval SS34 rotor at 5,000 r.p.m. (4°C) for 10 min. The supernatant was recovered and used as the CD9 antigen preparation. Antigen solution (200 µl) was used to coat each well of the ELISA plate (Nunc) at 4°C for 24 hours with gentle shaking. The wells were then washed with excess PBS containing 0.05% Tween 20. The rabbit polyclonal antibody was serially diluted in PBS containing 0.05% Tween 20 and 0.1 mg/ml bovine serum albumin (BSA). Diluted antibody (200 µl) was added to the CD9-coated wells in duplicate. Similarly diluted pre-immune serum was also included as a negative control. The plate was incubated at 4°C overnight and the wells were washed as described above. Goat anti-rabbit IgG conjugated to alkaline phosphatase (200 µl) (Bio-Rad, Richmond, California, diluted 1/400 in PBS containing 0.5% Tween 20) was added to each well and the plate was incubated at room temperature for 2 hours, after which the wells were washed again as described above. The chromogenic substrate p-nitrophenyl phosphate was finally added (200 µl/well) and the plate was analyzed at room temperature with a microplate autoreader (EL309 Bio-tek Instrument, Inc., Burlington, Vermont) one hour later.

4. *^{125}I-Labelling of Cell Surface Proteins*

The iodination of cell surface proteins was performed according to the procedure described by Markwell and Fox (1978). In short, IODO-GEN (Pierce, Rockford, ILL) was solubilized at 1 mg/ml in chloroform and 50 µl were used to coat a precleaned glass tube under a stream of N_2. Then, 1 x 10^7 viable cells, or 1 x 10^9 fresh platelets in 0.5 ml PBS were transferred to the tube and iodination was initiated by addition of 500 µCi carrier-free Na^{125}I. The suspension was gently rotated for 10 min at room temperature and the reaction was terminated by addition of unlabeled NaI for a final concentration of 5 mM. The cells were then sedimented by centrifugation in an IEC Centra - 7R™

clinical centrifuge at 3,000 r.p.m. at room temperature for 10 min and washed with PBS until the supernatant fluid was free of $Na^{125}I$.

5. *Intrinsic Labelling of Cellular Proteins with ^3H-leucine*

Cells were intrinsically labeled when they were still in semi-log phase of growth, as described by Seehafer et al. (1984b). Briefly, 150 µCi of ^3H-leucine (55 Ci/mmol) (New England Nuclear, Boston, MA) was added to the cultures in 1 ml of leucine-free RPMI medium, supplemented with 0.5% FCS. After 4 hours of incubation in the cell culture incubator, the cultures were washed with Tris-buffered saline (TBS) at pH 7.4 and lysed with 0.5% Nonidet P-40 (NP-40), 1 mM Na_2HPO_4 in 20 mM Tris HCl (pH 8.0) in the presence of the protease inhibitors phenylmethylsulphonyl fluoride (1 mM) and chloromethyl-L-(2-phenyl-1-p-toluenesulphonamido) ethyl ketone (0.5 mM). Lysates were incubated on ice for 20 min and clarified by centrifugation in a Sorval SS34 rotor at 10,000 r.p.m. (4°C) for 15 min.

6. *Immunoprecipitation and SDS-PAGE*

Cells were solubilized at 1 x 10^6 cells in 0.3 ml (or in the case of platelets, 1 x 10^7 cells in 0.3 ml) of buffer containing 20 mM Tris-HCl pH 8.0, 1 mM Na_2HPO_4, 0.137 M NaCl and 0.5% NP-40. The cell lysates were clarified by spinning in an Eppendorf centrifuge (15,000 r.p.m.) at 4°C for 15 min. For each immunoprecipitation sample, 30 µg of antibodies were added per 0.3 ml lysate. The solution was rotated at 4°C for 2 hours in an Eppendorf micro-centrifuge tube after which 40 µl of reconstituted Staphylococcus protein A-Sepharose 4B beads (Pharmacia, Uppsala, Sweden) were added, as outlined by Seehafer et al. (1984b). The beads were reconstituted with buffer used for cell lysis (0.01 gms/150µl) at room temperature for 30 min before use. Following another 1 hour rotation at 4°C, the lysate-beads mixture was washed 5 times with 0.5 ml of buffer containing 0.1 M Tris-HCl pH 8.0, 0.5 M LiCl, and 0.5% NP-40 and once with 0.01 M Tris-HCl pH 6.8. All solutions were kept on ice and each

centrifugation was performed for 30 sec using an Eppendorf micro-centrifuge placed in a cold room. Proteins were separated from the protein A-Sepharose 4B beads by the adding 30 µl of buffer containing 2% SDS, 5% 2-ME, 0.01 M Tris-HCl pH 6.8, 10% glycerol and 0.003% bromophenol blue to the well drained pellets and boiling for 5 min. The supernatant was then loaded onto 12.5% regular or 5% - 20% gradient SDS-polyacrylamide slab gels prepared and run according to the method of Laemmli, (1970). Regular or pre-stained protein size markers (Bio-Rad, Richmond, California) were included for calibration. To visualize the radiolabeled proteins, the gel was treated with 20% 2,5-diphenyloxazole in glacial acetic acid (w/v) for 60 min and then 2.5% glycerol and 0.01 M Na-Phosphate pH 7.2 for another 60 min before it was dried in a slab gel drier, and used to obtain a fluorographic image on pre-flashed Kodak XAR-5 film. Unlabeled proteins were visualized either by staining wet gels with Coomassie Brilliant Blue or with ammoniacal silver.

7. *Coomassie Brilliant Blue and Silver Stainings*

Coomassie Brilliant Blue staining of protein bands was carried out according to the procedure of Meyer and Lamberts (1975). Polyacrylamide gels were first rinsed with H_2O and fixed for 20 min at room temperature in a solution containing 10% TCA, 10% acetic acid and 30% methanol. Staining was carried out for 60 min at room temperature in 0.2% Coomassie Brilliant Blue (Fisher) prepared in a solution of 10% acetic acid and 40% methanol. Destaining was performed over a period of 4 hours with several changes of 10% acetic acid/ 40% methanol.

Whenever stained protein was to be recovered from polyacrylamide gels, the Coomassie Brilliant Blue staining regimen was modified as described by Hunkapiller et al. (1983). Briefly, a 10% (w/v) stock solution of Coomassie Brilliant Blue G 250 (research grade, Serva, New York) was prepared and cleared through a Whatman No. 3 filter before use. Unfixed polyacrylamide gels were first rinsed in H_2O and then stained

at room temperature for 20 min with a 0.5% solution of filtered Coomassie Brilliant Blue prepared in acetic acid-isopropyl alchohol-water (1:3:6 v/v/v), with gentle shaking. Destaining was carried out for 2-3 hours at 4°C with gentle shaking in a buffer containing acetic acid-methanol-water (50:165:785 v/v/v). Absorbent paper towels (Kimwipes) were placed in the corners of the destaining chamber to adsorb the excess dye as it eluted from the gel.

Silver staining kits were purchased from Bio-Rad and the staining regimen followed in the protocol provided. Gels were first fixed once in 40% methanol / 10% acetic acid for 60 min and twice for 30 min each in 10% ethanol / 5% acetic acid. Freshly prepared "oxidizer" reagent (Bio-Rad) was then added and the gels were gently rotated for 10 min, followed by three washes in H_2O. The gels were then immersed in freshly prepared "silver" reagent (Bio-Rad) for 30 min. After a final rinse in H_2O, the gels were developed in "developer" solution (Bio-Rad) for 5 to 10 min. Several changes of developer solution were required to obtain optimal resolution. The staining reaction was then terminated with 5% acetic acid.

8. *Western Blotting*

Proteins were transferred to nitrocellulose membranes by both "capillary" and "electro-blotting". Capillary-blotting of protein gels was performed using methodology similar to that commonly used in the transfer of DNA/RNA (Maniatis et al., 1982). Typically, a gel was placed atop a piece of 'Saran-wrap' resting on a glass plate. A cut-to-size nitrocellulose filter (0.45 µm, Gelman Sciences Inc. Ann Arbor, MI) pre-wetted with PBS was then layered onto the gel, followed by two layers of Whatman 3 mm paper (pre-wetted with PBS), and one layer of dry Whatman 3 mm paper. A stack of paper towels (approximately 15 cm in height) was finally added with 1 kg weight. The proteins were allow to transfer by capillary action to nitrocellulose membranes at room temperature overnight. When required, the gel was sandwiched between two layers of

nitrocellulose membranes and blotting papers placed on top and underneath the gel. At the end of transfer, the orientation of the gel as well as the lane origins were marked and the filter was peeled off the gel. The filter was washed once with PBS and incubated with appropriate dilutions of mAbs overnight at 4°C. For polyclonal antibodies, the filters were first incubated with 20% FCS to block non-specific binding sites on the filter before incubating with the antibody. After washing three times with PBS, the filters were incubated for 2 hours at 4°C with goat anti-mouse or anti-rabbit IgG horse radish peroxidase conjugated secondary antibodies (Bio-Rad). After two more washes in PBS, the protein bands were visualized by developing the filters in the chromogenic substrate reagent provided by the Bio-Rad staining kits. Pre-stained protein size markers used for size calibration were purchased from Bio-Rad.

The trans-blot electrophoretic blotting apparatus (trans-blot cell) was purchased from Bio-Rad. Proteins were blotted onto the nitrocellulose filter according to the protocol provided. Typical transfers were carried out at 75 volts using a Model 250/2.5 power supply (Biorad) with constant stirring overnight at 4°C in a buffer containing 25 mM Tris-HCl pH 7.5, 292 mM glycine, and 20% methanol. Proteins electro-transferred to nitrocellulose filters were visualized as described above.

9. *Partial Proteolytic Cleavage of Polypeptides*

Radiolabeled CD9 protein was immunoprecipitated by 50H.19 mAb, or by CD9 polyclonal antibody, fractionated on by SDS-PAGE to remove the co-precipitates, and the gel strips containing the gp22 component were excised with the aid of autoradiography. The protein was then digested with S. aureus V8 protease using the limited proteolysis procedure described by Cleveland et al., 1977. Gel pieces were washed for 20 min in 0.01 M Tris-HCl, pH 6.8, 1 mM EDTA, 0.1% SDS and digested during electrophoresis with the protease at 100 μg/ml. Separation of the fragments was performed on 16% SDS-polyacrylamide slab gels.

10. *Peptide Synthesis*

The amino acid sequence predicted from the CD9 cDNA clone was analyzed by the Wisconsin DNA analysis program at the Department of Medical Genetics, University of Toronto (Toronto), and by the protein analysis program at the Department of Biochemistry, University of Alberta (Edmonton) and used to design peptides corresponding to two of the hydrophilic regions of the CD9 protein. Both peptides were synthesized by the Alberta Peptide Institute on an Applied Biosystems Peptide Synthesizer (Model 430A) utilizing the t-Boc Na-protection and benzyl type side-chain protection synthesis methods. The purified peptides were then coupled to bovine serum albumin and to Keyhole Limpet Hemocyanin carrier proteins and the carrier-coupled peptides were resuspended in 10mM ammonium bicarbonate buffer.

C. LARGE SCALE PURIFICATION OF CD9 ANTIGEN

1. *Preparation of Cell Lysates*

Suspension culture of NAML-6 cells were initiated at a density of 1×10^5 cells/ml in 150 ml tissue culture flasks containing 100 ml of 10% RPMI medium. The flasks were laid flat in the incubator and cultured for 4 to 5 days until the cell concentration reached 1×10^6 cells/ml. 100 ml of fresh 10% RPMI medium was then added to each flask, and the culture was continued for 3 to 4 more days until a density of 2×10^6 cells/ml was obtained. Cell viability was verified by the Trypan blue dye exclusion test. Saturated cultures were harvested by centrifugation in 200 ml bottles in a Sorval GAS rotor at 800 r.p.m. (4°C) for 5 min. The cell pellets were pooled into 50 ml polypropylene tissue culture tubes (Corning) and washed three times with ice-cold PBS before they were lysed in the buffer used for cell lysis in immunoprecipitation (3×10^8 NALM-6 cells/ml, or 3×10^9 platelets/ml; see Section B6). After removing the cell

debris, the lysates were either stored at -70°C for later use, or processed immediately to avoid protein degradation.

The cell lysates were clarified by centrifugation in a Sorval SS34 rotor at 10,000 r.p.m. (4°C) for 15 min. The supernatant was recovered, to which 3.5 M NaCl was added to bring the salt concentration to 0.5 M and incubated with 1/50 volume of Pansorbin (10% suspension of heat killed and formalin fixed *Staphylococcus aureus* Cowan I strain, Boehring Diagnostics, La Jolla, CA) at 4°C for 30 min with gentle shaking. The bacteria were washed twice in PBS before use to remove trace amount of formalin. Once the lysates were clarified by centrifugation in a Sorval SS34 rotor at 5,000 r.p.m. (4°C) for 15 min, the lysates were ready for use in mAb affinity chromatography.

2. *Cross-Linking of mAb 50H.19 to Protein A-Sepharose*

Protein A-Sepharose CL-4B (Pharmacia) was mixed with gentle shaking for 30 min at room temperature with a saturating amount of mAb 50H.19 in 40 ml of 0.1 M borate buffer pH 8.2 in a 50 ml cell culture tube. The Sepharose beads were recovered by centrifugation in the IEC Center - 7R™ clinical centrifuge at 3,000 r.p.m. for 5 min, washed with excess borate buffer and finally with 0.2 M triethanolamine, pH 8.2. The beads were next resuspended in 20 volumes of 80 mM dimethylpimelimidate dihydrochloride (Pierce) freshly prepared in 0.2 M triethanolamine (pH readjusted to 8.2) and agitated gently at room temperature for 45 min. The reaction was terminated by centrifugation at 3,000 r.p.m. for 5 min and resuspending the beads in an equal volume of 80 mM ethanolamine (pH 8.2) for 5 min. The beads were then recovered by centrifugation and washed with excess borate buffer. Beads not used immediately were resuspended in borate buffer supplemented with 0.02% sodium azide.

3. *mAb 50H.19 Affinity Chromatography*

The Pansorbin-adsorbed cell lysate was added to the antibody cross-linked Sepharose beads in a 50 ml cell culture tube and the tube was rotated in a cold room (4°C) overnight to allow the antigen to bind to the cross-linked antibody. The beads were recovered by centrifugation in the ICE Centra - 7R^{TM} centrifuge at 3,000 r.p.m. (4°C) for 5 min and rinsed extensively in buffer containing 0.5 M NaCl, 0.05 M Tris-HCl pH 8.2, 1 mM EDTA and 0.5% NP-40. The beads were then packed into a disposable 8 ml econo-column (Bio-Rad) with a Pasteur pipet. Flow-through fractions were discarded. The antigen was eluted from the column as 1 ml fraction with 0.05 M diethylamine (pH 11.5) and 0.5% deoxycholate (or 0.5% NP-40) into Eppendorf micro-centrifuge tubes containing 0.1 ml 0.5 M NaH_2PO_4 . Twenty fractions were collected for each elution. The tubes containing the antigen were identified by analyzing 10 µl of solution from each tube by SDS-PAGE followed by Coomassie Brilliant Blue or silver staining.

The column was reconstituted by washing with excess 0.1 M acetate buffer pH 4.0 containing 0.5 M NaCl, alternating with 0.1 M Tris-HCl pH 8.0 and 0.5 M NaCl. The beads were then rinsed twice with the borate buffer before use or stored at 4°C in borate buffer containing 0.02% sodium azide.

4. *Dialysis of the Column Eluate*

Dialysis tubing (Spectrapor membrane tubing, Spectrum Medical Industries, Inc. Los Angeles, molecular weight cut-off 12,000) was prepared by boiling in 1% $NaHCO_3$ for 30 min followed by extensive washing in H_2O. The column eluate was dialyzed extensively against either H_2O, or 0.01 M Tris-HCl pH 7.2.

Alternatively, an ultra-filtration apparatus (Model 8050, Amicon Canada, Ltd, Oakville, Ont.) equipped with a YM10 filter (No. 13622, 43 mm with a molecular cut-

off of 10,000 Daltons, Amicon) was used for pressure dialysis and concentration of the column eluate against 0.01 M Tris-HCl pH 7.2 under 55 psi N_2.

5. *High Performance Liquid Chromatography*

High performance liquid chromatography (HPLC) to further purify the CD9 antigen was carried out on a Varian machine (Model 5000) equipped with a reverse-phase C18 column. The column was equilibrated with 0.1% trifluoroacetic acid and the proteins were eluted in 0-70% acetonitrile gradient. Samples were collected into Eppendorf micro-centrifuge tubes using a fractional collector (Pharmacia FRAC-100) and analyzed by measuring the absorbance at 280 nm.

6. *Electroelution of CD9 Protein*

Preparative SDS-PAGE was performed using a 32 cm long slab gel with large amounts of CD9 antigen isolated from the mAb affinity chromatography. Protein size markers were loaded on both sides of the samples. The gel was stained and destained with Coomassie Brilliant Blue as described before. The band containing the CD9 antigen was excised with a razor blade, rinsed with several changes of H_2O for 2 hours and then either processed immediately or stored at -20°C for later use. Electro-elution was carried out according to the method of Hunkapiller et al. (1983). Dialysis disks were prepared by soaking dialysis tubing (similar to that used in dialyzing the column eluate) in 1 % $NaHCO_3$ for 1 hour at 60°C, followed by washing with H_2O, soaking in 1% SDS for 1 hour at 60°C and a final washing step with H_2O. The tubing was cut into disks with a sharp cork borer, rinsed with H_2O and store at room temperature in 0.1% SDS / 0.1% sodium azide. The soaked gel was then placed in a petri dish, covered with 10 to 20 ml of H_2O and diced into 1 mm cubes without crushing the gel. After removing the water with a Pasteur pipet and blotting with Kimwipe towels, the diced gel was soaked in buffer containing 0.1% SDS in 0.05 M NH_4HCO_3 for 5 min before being transferred into the wells of the elution apparatus (Tyler Research Instruments Ltd., Edmonton,

Alta) already fitted with the disks. 2% SDS in 0.4 M NH_4HCO_3 supplemented with 0.1% fresh DTT was used to cover the diced gel in the wells, to which elution solution was overlaid to fill the wells. Bubbles formed under the elution well caps were removed by a syringe with a bent needle. After soaking for 3 to 5 hours, the electro-elution was performed for 12 to 16 hours at 50 volts. The elution solution was then replaced with 0.02% SDS in 0.01 M NH_4HCO_3 and electro-elution-dialysis was continued at 80 volts for another 20 to 24 hours. The proteins were then recovered from the wells with a 1 ml syringe and the wells were rinsed with 0.1 ml of the buffer used for dialysis. The protein samples (including the rinse material) were pooled into Eppendorf micro-centrifuge tubes from which only a fraction was used to determine the purity of the recovered protein by SDS-PAGE and Silver staining.

D. IMMUNIZATION OF ANIMALS WITH CD9 ANTIGEN

1. *Immunization of Rabbits with Column Eluates*

Fractions eluated from the mAb affinity chromatography that contained CD9 antigen were used to immunize the rabbits. SDS (0.01%) and 2-ME (10 mM) were added to the antigen preparation which was then boiled for 10 min and cooled on ice. Saline was added to the antigen preparation to bring the volume to 1 ml. An equal volume of Freund's adjuvant was then added and the preparation was emulsified by drawing through a 18 gauge needle and then injected subcutaneously into rabbits. A boosting injection was done 3 weeks before every bleeding. Blood was collected from the central ear vein. Pre-immune serun was obtained on the day of the first injection and was used as negative control for antisera positivity against CD9 antigen. During the immunization period, 5 ml of whole blood was obtained before each injection to test the positivity of the antisera. The blood was allowed to clot at room temperature for two hours after which the clotted blood was detached from the tube wall, and the tubes were

left at 4°C overnight to achieve a better clot retraction. The supernatant was decanted into a centrifuge tube and the serum was separated from the blood cells by centrifugation in the IEC Centra - 7R™ centrifuge at 3,000 r.p.m., 4°C for 10 min, and then analyzed by ELISA and/or Western blotting, as described before. Once high titres of antisera were produced by the rabbit, 30 ml blood samples were collected. The antisera were aliquoted into Eppendorf micro-centrifuge tubes and stored at -20°C.

2. *Immunization of Rabbits with Synthetic Peptides*

For priming, each rabbit received 500 μg of synthetic CD9 peptide coupled to the Keyhole Limpet Hemocyanin (KLH) carrier. The peptide was mixed with an equal volume of Freund's complete adjuvant (1.1 ml total volume), emulsified as described above, and injected at multiple subcutaneous sites. A boosting injection (250 μg of synthetic peptide in incomplete Freund's adjuvant per rabbit) was performed exactly two weeks later followed by repeated boosting every two weeks after the previous injection. Pre-immune serum collection and the subsequent evaluation of the developing antibody titres were conducted as previously described, except for the fact that BSA-conjugated peptides were used as antigens for detecting the presence of CD9-reactive antibody in the ELISA assay.

E. ISOLATION AND CHARACTERIZATION OF RNA

1. *Large Scale Isolation of Total Cellular and Poly A$^+$ RNA*

One to 5 x 10^8 cells were washed twice in ice-cold sterile PBS, and pelleted by centrifugation in a Sorval SS34 rotor at 3,000 r.p.m. (4°C) for 10 min. Total cellular RNA was isolated by homogenizing the cell pellets in 3 to 6 ml of 4 M guanidinium thiocyanate (BRL), 0.5% sodium N-lauroylsarcosine, 25 mM sodium citrate (pH 7.0), 0.1 M 2-ME, and 0.1% Sigma antifoam A (Chirgwin et al., 1979) using a Sorvall single-blade homogenizer, (three 30 second cycles with constant cooling on ice). The

homogenate (3 ml) was layered over a 2 ml cushion of 5.7 M CsCl, 0.1 M EDTA pH7.0 and the RNA was pelleted by centrifugation in a Beckman SW 50.1 swinging bucket rotor at a speed of 36,000 r.p.m. (20°C) for 12 hours. The supernatant was carefully discarded and the pellets were rinsed quickly but gently with ice cold, sterile RNAse-free H_2O. The RNA pellets were dissolved by heating (42°C), shaking, and vortexing in 1 ml of 25 mM EDTA/0.1%SDS. RNA solution was clarified by centrifugation in a Sorvall SS34 rotor at 4,000 r.p.m. (4°C) for 10 min. One tenth volume of 3M sodium acetate (pH 5.3) was added to the supernatant, followed by 2.5 volumes of 95% ethanol. The RNA was precipitated at -70°C overnight. The RNA pellet was collected by centrifugation at 12,000 r.p.m. (4°C) for 30 min, dried in the Speed-Vac, and dissolved in 0.5-1 ml of 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.1% SDS. LiCl was added to a final concentration of 0.5 M, and Poly A^+ RNA was isolated from this material by three consecutive cycles of adsorption and elution from oligo-dT cellulose (Collaborative Research), using 0.5 M LiCl for loading, 0.1 M LiCl for washing, and LiCl free solution for eluting the column, as described by Bleakley et al. (1981). The poly A^+ fraction was precipitated twice with sodium acetate and ethanol as described above, before being finally resuspended in sterile RNAse-free H_2O. This material was used directly for generating cDNA libraries, and for Northern blotting.

2. *Small Scale Isolation of Cytoplasmic RNA for Dot Blot Analysis*

Cytoplasmic RNA was isolated essentially as described by White and Bancroft (1982). One to 5 x 10^6 cells were washed twice in ice-cold sterile PBS, resuspended in 1.5 ml PBS, and transferred to Eppendorf micro-centrifuge tubes placed on ice. The cells were pelleted for 15 sec using an Eppendorf micro-centrifuge. The supernatant was removed and the cell pellet was resuspended by vortexing in the remaining trace amount of PBS. Ice-cold 10 mM Tris-HCl pH 8/1 mM EDTA (TE; 45 µl) was added to the resuspended pellet, followed 5 min later by 25 µl of 5% NP-40. The cell nuclei were

removed by centrifugation for 2 min using an Eppendorf micro-centrifuge. The supernatant (50 µl) was recovered and transferred into a tube already containing 30 µl of 20 x SSC (1 x SSC: 150 mM NaCl and 15 mM sodium citrate, pH 7.0) and 20 µl of 37% formaldehyde (J.T. Baker Chemical Co., Phillipsburg, NJ). The RNA was further denatured by heating to 60°C for 15 min, and was stored at -70°C until before it was applied to filters.

For Dot Blot analysis, total cellular RNA was denatured and serially diluted in 15 x SSC in microtiter plate wells. Each diluted sample was then transferred to the filtration cells of a Dot-Blot apparatus (Bio-Rad) housing a nitrocellulose filter (Schleicher & Schuell BA 85; pre-wetted in water for 5 min, then in 15 x SSC for 1 hour), and drawn through slowly according to the manufacturer's protocol. The nitrocellulose filter was removed while still under suction, air dried for 1 hour, sandwiched loosely between two layers of Whatman 3MM paper, and baked for 1.5 hours at 80°C in a vacuum oven in order to "fix" the RNA. The filter was either used immediately or was stored under vacuum at room temperature for later use.

3. *RNA Gels and Northern Blotting*

RNA was separated on denaturing gels and blotted directly onto nitrocellulose using routine methodology (Thomas, 1980). The electrophoresis buffer [20 mM 3-(N-morpholino) propane sulfonic acid pH 7.0 (MOPS, Sigma), 5 mM sodium acetate, and 1 mM EDTA] was recirculated with a peristaltic pump during electrophoresis (Maniatis et al., 1982). Agarose gels (0.8%) were made up in electrophoresis buffer and supplemented with 0.67% formaldehyde and 1.0 µg/ml ethidium bromide. RNA samples were denatured by heating at 55°C for 15 min in 20 µl of electrophoresis buffer containing 6.5% formaldehyde and 50% deionized formamide (Fluka, Germany). The samples were chilled on ice and 2 µl of RNA loading buffer (50% glycerol, 1 mM EDTA without dye) was added to each sample prior to loading. The progress of the

electrophoresis was monitored by following the migration of a dye marker (0.25% bromophenol blue prepared in loading buffer) loaded in a sample well which did not contain RNA. Typically, gels were run until the dye front had advanced two-thirds of the length of the gel. Gels were rinsed for 30 to 60 min in 20 x SSC (in order to remove excess ethidium bromide), photographed, and transferred by capillary (Northern) blotting to nitrocellulose filters. The membranes were pre-wet with 10 x SSC prior to transfer, and the blotting was carried out without the use of a wick according to the method outlined by Maniatis et al. (1982). Following the transfer, the lane origins were clearly marked on each filter. the blots were processed as described above in the dot blotting section.

F. ISOLATION AND CHARACTERIZATION OF DNA

1. *Isolation of Plasmid DNA*

For rapid plasmid DNA preparation (Birnboim and Doly, 1979), individual bacterial colonies were selected with sterile tooth picks and inoculated separately into 5 ml of Luria-Bertani medium (LB medium) supplemented with appropriate antibiotics. The cultures were allowed to grow for 4 to 6 hours in a bacterial "dry" incubator (37°C) with constant shaking (200 r.p.m.), after which 1.5 ml were removed for DNA preparation. The remainder of the cultures was numbered and stored at 4°C for later use. The bacteria were collected in Eppendorf tubes (1.5 ml capacity) by centrifugation for 60 sec in an Eppendorf micro-centrifuge, and the pellets were resuspended by vortexing in 0.1 ml of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0 containing 4 mg/ml of lysozyme. Five min later, 200 µl of freshly prepared 0.2 N NaOH and 1% SDS were added, and each tube was inverted rapidly to mix the solution and held on ice for 5 min. Ice-cold 3 M sodium acetate, pH4.8 (150 µl) was then added, and each tube was held on ice for an additional 5 min. The cell debris was removed by centrifugation in an

Eppendorf micro-centrifuge for 5 min (4°C) and the supernatants were decanted into fresh micro-centrifuge tubes. The tubes were then filled with 95% ethanol, mixed and left on ice for 5 min. The DNA was pelleted by centrifugation in an Eppendorf micro-centrifuge for 5 min (4°C). The DNA pellet was resuspended in 100 µl of 0.3 M sodium acetate, pH 7.0 and re-precipitated with 200 µl 95% ethanol. After washing with 70% ethanol, the pellet was briefly dried in Speed-Vac and dissolved in 50 µl of sterile H_2O. The plasmid DNA was then ready for analysis by restriction enzyme digestion and by agarose gel electrophoresis. If the plasmid DNA was intended for cloning, 50 ml of LB culture was used instead, and the DNA was extracted twice with phenol and twice with ether before being dissolved in 0.3 M sodium acetate and precipitated.

For large scale plasmid preparation (Clewell and Helingski, 1972), 1 liter of LB culture (containing the appropriate antibiotics) was inoculated with a fresh 5 ml overnight bacterial culture. The culture was allow to grow overnight in the bacterial incubator (37°C) with constant shaking (200 r.p.m.). The bacteria were pelleted in 250 ml centrifuge bottles by centrifugation in Sorvall GSA rotor at 7,000 r.p.m. (4°C) for 10 min. The bacterial pellet was then resuspended in 4 ml of sucrose buffer (15% sucrose, 50 mM Tris-HCl pH 8.0, and 50 mM Na_2EDTA) to which 10 mg of lysozyme were added. Cell lysis was allow to proceed at room temperature for 10 min after which 5 ml of Triton solution (0.4% Triton X-100, 0.05 M Tris-HCl pH 8.0 and 50 mM Na_2EDTA) were added and the lysate was left to stand at room temperature for an additional 10 min. Cell debris was removed by centrifugation in a Sorvall SS34 rotor at 10,000 r.p.m. for 10 min. The supernatant was then decanted into a test tube and the volume adjusted to 8 ml with TE (10 mM Tris-HCl pH 7.4, 1 mM EDTA) buffer. CsCl (8 gm) was added and the DNA-CsCl solution was transferred to 16 ml quick-seal ultracentrifuge tubes. Each tube was then overlaid with a 0.35 ml layer of 15 mg/ml ethidium bromide solution. The tubes were sealed and the plasmid DNA was banded by

centrifugation in a Beckman Ti 75 rotor at 55,000 r.p.m. (room temperature) for 20 hours. Plasmid DNA was harvested as described in Davis et al. (1986). The ethidium bromide was removed by extraction with CsCl saturated isopropanol, and the CsCl was removed by extensive dialysis against TE (10 mM Tris-HCl pH 7.4, 1 mM EDTA) at 4°C. DNA isolated by this method was used for subcloning, for polybrene-mediated DNA transfection, and for DNA microinjection.

2. *Isolation of Phage DNA*

Growth, maintenance, and preservation of bacteriophages were essentially as described by Davis et al. (1980) and Maniatis et al. (1982), except that the LB medium was routinely supplemented with 0.2% glucose, 10 mM Tris-HCl pH 7.5, and 2 mM $MgCl_2$ after autoclaving. For rapid bateriophage DNA isolation, selected phage plaques were picked with Pasteur pipets, and transferred into Eppendorf tubes containing 1 ml of λ diluent (10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.1 mM Na_2EDTA) and a few drops of chloroform. The phage particles were allowed to elute from the agar at 4°C overnight, after which they were titred individually. 1 x 10^6 plaque forming unit (p.f.u.) of phage were used to infect 0.1 ml of fresh log phase E. coli Y1090 cells ('plating stock', grown in 0.2% maltose and resuspended in λ diluent at an $O.D._{600}$ of 2.4) at room temperature for 30 min. The phage were spread, using 3 ml of 'top agar', on LB agar medium (pH 7.5) previously dispensed in 90 mm Petri dish plates. The plates were incubated at 42°C for 8 to 10 hours in the bacterial incubator until the phage plaques were confluent. The plates were then cooled to 4°C and 10 ml of cold λ diluent plus a few drops of chloroform were added. The plates were gently shaken on a rotating platform for 10 hours at 4°C after which the supernatant was recovered and clarified by centrifugation in a Sorval SS34 rotor at 5,000 r.p.m. (4°C) for 10 min. After transferring the supernatant to a fresh tube, 5 μg/ml each of RNase A and DNase I (both were from Pharmacia, Uppsala, Sweden) were added and the solution was incubated at

37°C for 30 min. An equal volume of ice cold 20% PEG (polyethylene glycol 8000) and 2 M NaCl in λ diluent was then added, and the solution was left on ice for 2 hours to precipitate the phage. The phage particles were pelleted in a Sorval SS34 rotor at 10,000 r.p.m. (4°C) for 20 min and were next dissolved by vortexing in λ diluent and centrifuged at 5,000 r.p.m. for 10 min to remove insoluble materials. The supernatant was recovered, to which 10 mg/ml of proteinase K (Boehringer Mannheim, Dorval, Quebec) was added. The tube was incubated at 37°C for 30 min, followed by the addition of 1/100 volume of 10% SDS, and another 30 min incubation at 37°C. The reaction was terminated by adding 1/100 volume of 0.5 M EDTA and incubating at 70°C for 15 min. The proteins were removed by two rounds of phenol and ether extraction. The phage DNA was precipitated by the addition of an equal volume of isopropanol, and incubation on dry ice for 30 min. After centrifugation in a Sorval SS34 rotor at 10,000 r.p.m. (4°C) for 15 min, the DNA pellet was washed once with 70% ethanol, dried briefly in Speed-Vac, and resuspended in TE buffer for analysis by restriction enzyme digestion.

The bacteriophage DNA used for the construction of cDNA libraries was isolated from wild type λgt11 phage by a different method. Twenty eight individual blue plaques (β-galactosidase positive; wild type) were picked from agar plates used to initiate a stock of λgt11 phage using commercially available phage DNA arms (Vector cloning system, San Diego, CA). Each phage was placed in a separate tube containing 1 ml of λ diluent plus a few drops of chloroform. The phage was individually titred, plated out, grown, recovered, and clarified as described above, except for the fact that 2×10^6 p.f.u. were used to infect 0.3 ml of plating stock which was later plated out on the large 150 mm Petri dish plates using 6 ml of 'top agar' per plate. CsCl (BRL, ultrapure) (214.4 gm) was dissolved in 268 ml of the phage supernatant (67 gm CsCl per 82 ml of λ diluent yields a final volume of 100 ml with a density of 1.5 gm/ml) and the solution was loaded

into 8 quick-seal tubes (39 ml capacity, Beckman) and centrifuged in a Beckman Ti70 rotor at 40,000 r.p.m. (4°C) for 35 hours. The clear blue band representing the phage near the center of each tube was removed with a syringe, pooled, and an equal volume of λ diluent was added. The recovered material was then gently overlaid (6 ml/tube) atop a pre-formed CsCl step gradient [consisting of 3 ml of CsCl in λ diluent (density 1.4 gm/ml) over a 2.25 ml cushion of CsCl in λ diluent (density 1.6 gm/ml)]. The tubes were centrifuged in a Beckman SW 40.1 swinging bucket rotor at 26,000 r.p.m. (20°C) for 2 hours. The phage bands were collected, pooled and 0.6 gm of CsCl was added per ml of liquid. The sample was placed at the bottom of a fresh ultracentrifuge tube (6ml/tube) and overlaid with 3 ml of CsCl solution (density 1.6 gm/ml) followed by 3 ml of CsCl solution (density 1.4 gm/ml). The tubes were centrifuged as above and highly purified phage particles were collected. Tris-HCl pH 8 was added to a final concentration of 50 mM and EDTA to a final concentration of 20 mM. An equal volume of deionized formamide was added, and the mixture was allowed to stand at room temperature for 3 hours. One volume (*i.e.* equal to the volume of formamide) of water was added, followed by 6 volumes of ethanol. The DNA was precipitated at -20°C for several hours and collected by spooling onto a glass rod. The precipitate was rinsed several times in 70% ethanol, dried, and resuspended in TE buffer. The purified phage DNA was then cleaved with Eco RI (New England Biolabs), extracted twice with phenol, twice with ether, and precipitated by ethanol. This procedure was repeated to ensure that all DNA was cut to completion.The cleaved DNA was next resuspended in water, heated to 70°C for 5 min, incubated at 42°C for 30 min (to anneal the cohesive ends on the phage arms) and chilled on ice. ATP and DTT were added to final concentrations of 1 mM and 10 mM respectively, and the phage arms were stored frozen as 25 µl aliquots at -70°C.

3. *Isolation of Genomic DNA from Whole Tissues*

Human placenta and thymus specimens were obtained fresh from the University Hospital, University of Alberta (Edmonton). Once residual blood was removed by blotting and washing with sterile PBS, the tissues were placed in a sterile Petri dish and minced into 1 to 5 mm^3 pieces with a surgical blade and scissors. Care was taken to remove as much connective tissue as possible. For a typical DNA extraction, 3 to 5 grams of minced tissue were pulverized with a pestle in a mortar partially filled with liquid nitrogen. The finely ground tissue was then poured into 50 ml cell culture tubes. The liquid nitrogen was allow to evaporate at room temperature and 10 ml of 10 mM Tris-HCl pH 7.4, 10 mM NaCl, and 25 mM EDTA were added to each tube. The powdered tissue was resuspended by inverting the tubes several times. Genomic DNA was released by treatment with detergent and proteinase K as described by Davis et al. (1986). Briefly, 1 ml of 10% SDS was added to each tube followed by 11 mg of proteinase K powder. The tubes were incubated at 37°C for 1 hour after which an additional 11 mg of proteinase K was added, and the incubation continued for another 1 to 2 hours until the tissues were completely dissolved (when the solution became viscous and clear). The salt concentration of the DNA solution was adjusted by the addition of 1 ml of 5 M NaCl. The proteins were removed by two rounds of extraction with phenol and ether. High molecular weight genomic DNA was precipitated with 2.5 volume of 95% ethanol pre-chilled at -20°C and collected by spooling on a glass rod. Residual DNA was recovered by centrifugation. The DNA collected by both methods were pooled, washed with 70% ethanol, dried briefly, and resuspended in TE buffer overnight at 4°C. DNA concentration and purity were determined by a spectrophotometer (where 1 $O.D._{260}$ = 50μg/ml DNA and an $O.D._{260/280} > 1.85$ were taken as standard values). Genomic DNA samples prepared in this manner were acceptable for analysis by Southern blotting.

4. *DNA Gel and Southern Blotting*

High molecular weight genomic DNA samples were digested to completion with selected restriction endonucleases, fractionated on 0.8% or 1% agarose gels, and blotted directly onto nitrocellulose membrane according to the methods described in Maniatis et al. (1982). Agarose gels were made up in TBE buffer (89 mM Tris-borate, 89 mM borate acid, and 2 mM EDTA) plus 1.0 μg/ml of ethidium bromide. The gels were run in TBE buffer until the bromophenol blue dye marker migrated approximately two thirds of the length of the gel, and were then photographed. The DNA was denatured by soaking the gels with constant stirring on a platform shaker in several volumes of 1.5 M NaCl and 0.5 M NaOH for one hour at room temperature. The gels were then neutralized under the same condition with several changes of 1 M Tris-HCl pH 8.0 and 1.5 M NaCl and finally soaked in 20 x SSC for one hour at room temperature. Nitrocellulose filters were pre-wet in 2 x SSC for 30 min at room temperature before use and capillary blotting was carried out overnight as outlined by Maniatis et al. (1982), using 10 x SSC as the transfer buffer. Following transfer, the positions of the gel slots were marked on the filter with a ball-point pen. The filters were then washed in 5 x SSC at room temperature for 5 min and processed for prehybridization.

G. NUCLEIC ACID PROBES AND HYBRIDIZATION CONDITIONS

1. *Oligonucleotide Probes*

Two oligonucleotide probes were synthesized and purified by the Alberta Heritage Foundation for Medical Research Regional DNA Synthesis Laboratory, University of Calgary (Calgary) on an Applied Biosystem nucleotide synthesizer. The oligonucleotide sequences were deduced from the presumed N-terminal amino acid sequence of the CD9 protein obtained by direct sequencing of the intact protein. The synthetic oligonucleotides were labeled at the 5' end using T4 polynucleotide kinase (Pharmacia,

16 u per reaction) in a 50 µl reaction volume containing 1.0 µg of oligonucleotide, 150 µCi of γ-P^{32}-ATP (New England Nuclear, > 3000 Ci/mmol), 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.1 mM spermidine, and 0.1 mM EDTA. The reaction was allowed to proceed for one hour at 37°C after which the labeled DNA was separated from unincorporated ^{32}P by chromatography through a Sephadex G-50 column prepared in a small Pasteur pipet plugged with glass wool. The labeled probes were eluted from the column with 0.1 M NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA, and 0.02% SDS.

2. *Labelling of cDNA Inserts*

DNA fragments of interest were generated by restriction enzyme cleavage, separated by agarose gel electrophoresis, and isolated from agarose gel slices by electroelution (Davis et al., 1986). Briefly, the gel bands containing the desired DNA fragments were excised and placed individually in dialysis bags containing just enough 0.2 x TBE buffer to cover the gels. The DNA was eluted from the gels by electrophoresis in 0.2 x TBE buffer at 300 volts for 3 to 5 hours at room temperature, after which the electrophoresis polarity was reversed and run for 1 to 2 min to dislodge the DNA from the walls of the dialysis bags. The entire contents of the dialysis bags were then transferred into Eppendorf micro-centrifuge tubes and agarose gel pieces were removed by centrifugation in an Eppendorf centrifuge for 15 sec. The supernatant (containing the DNA) was collected and extracted twice with phenol, twice with ether, and finally precipitated with ethanol as previously described. DNA fragments purified in this manner were used for radio-labelling by nick translation with α-^{32}P-dCTP (New England Nuclear, >3000 Ci/mmol) using a kit from Bio-Rad Laboratories (Bio-Rad, Richmond, CA).

Alternatively, DNA fragments were fractionated on 1% low melting point agarose (BRL) gels and were radio-labeled within the gel matrix by the random priming method

of Feinberg and Vogelstein (1983). Briefly, excised bands were placed in Eppendorf tubes, weighed, and an equal volume of sterile H_2O was added. The samples were then boiled for 5 min to melt the agarose, and the DNA solution was aliquoted in 33 µl volume (about 10 to 50 ng DNA) into Eppendorf tubes and stored at -20°C for later use. To label the DNA, a tube was boiled for 5 min to denature the DNA and cooled to 37°C in water bath for 10 min. Ten µl of OLB mixture [250 mM Tris-HCl pH 8.0, 25 mM MgCl2, 45 mM 2-ME; 1.0 M Hepes buffer pH 6.6, 100 µM dATP, 100 µM dGTP, 100 µM dTTP, 100 µM dCTP, and 540 µg/ml random hexadeoxyribonucleotides (Pharmacia)], 2 µl of BSA (10 mg/ml, Bio-Rad), 100 µCi of α-^{32}P-dCTP (New England Nuclear, >3000 Ci/mmol), and 5 units of Klenow (large fragment of DNA polymerase I, Bio-Rad) were added to the DNA containing tube and the reaction was allowed to proceed overnight at 37°C.

The labeled DNA was separated from unincorporated nucleotides by exclusion chromatography over Sepharose G50 as described above.

3. *Pre-hybridization, Hybridization, and Washing Conditions for Blots*

The nitrocellulose filters were washed in 4 x SSC prior to prehybridization. The filters were pre-hybridized at 42°C overnight in pre-hybridization buffer containing 50% formamide (v/v), 5 x SSC, 0.1%SDS, 1 mM ATP, 5 x Denharts solution, 200µg/ml sheared and denatured salmon sperm DNA, 200 µg/ml yeast tRNA, and 100 µg/ml poly A (Sigma). When oligonucleotide probes were employed, pre-hybridized filters were then washed 4 times in 4 x SSPE and hybridized at overnight 42°C in a solution identical to the pre-hybridization buffer except that it contained 20% formamide and 10% dextran sulfate (Pharmacia). When DNA probes of higher molecular weight were used, pre-hybridized filters were hybridized overnight at 42°C in a solution identical to the pre-hybridization buffer except that it contained 1 x Denharts solution (0.02% w/v each of ficoll, polyvinylpyrollidone, and BSA) and 10% dextran sulfate. The concentration of

^{32}P-labeled probes was 1 x 10^6 cpm/ ml for all hybridizations. Filters which had been hybridized with synthetic oligonucleotide probes were washed three times at 42°C for 15 min in 2 x SSC, 0.1% SDS. Filters hybridized to longer probes were washed three time at 55°C for 10 min in 2 x SSC and 0.1% SDS, and three times in 0.2 x SSC and 0.1% SDS. Filters were air dried, wrapped in Saran wrap, and exposed to Kodak X-Omat AR film under Cronex Hi-Plus intensifying screen.

H. CONSTRUCTION OF NALM-6 cDNA LIBRARIES IN BACTERIOPHAGE λgt11

1. *Reagents*

Vanadyl sulfate-nucleoside complexes (Vnx), an RNase inhibitor used in reverse transcription reaction was prepared by a method adapted from Berger and Birkenmeir (1979) and obtained from Drs. John Elliot and Vern Paetkau, Department of Biochemistry, University of Alberta (Edmonton). A kit containing the phage λgt11 arms, the E. coli strains Y1088, Y1089, and Y1090 was purchased from Vector Cloning System (San Diego, CA). λgt11 arms were also prepared as described previously in Section F2. The phage *in vitro* packaging extracts (Gigapacks) were obtained from Vector Cloning System as well. Eppendorf micro-centrifuge tubes were siliconized, rinsed extensively, and autoclaved prior to use.

2. *Library Construction*

The method used in the cDNA library construction in phage λgt11 was from the unpublished protocol of St. John, T., Rosen, J., and Gershenfeld, H (Department of Pathology, Stanford University), which was based on the procedure developed by Land et al., (1981).

a) First Strand cDNA Synthesis

The following reagents were combined, in the order given, in a siliconized tube placed on ice: 17.5 µl of water, 5 µl of 10 x salts [(1.0 M Tris-HCl (pH carefully

adjusted to 8.3 at 41°C), 0.5 M KCl, 0.1 M $MgCl_2$, and 0.1 M DTT)], 10 µl of all four dNTPs at 5 mM each (Pharmacia), 5 µl of oligo dT_{12-18} (1 mg/ml, Collaborative Research), 0.5 µl of 0.2 M Vnx, 5 µl of poly A+ RNA (2 mg/ml), and 2 µl α-^{32}P-dCTP (3200 Ci/mmol, New England Nuclear). 3.5 µl of AMV Reverse Transcriptase (17U/ml, Life Sciences) were then added, the contents were mixed by vortexing and incubated for one hour at 41°C. A second aliquot of 2.0 µl Reverse Transcriptase was added and the reaction continued for a further 90 min at 41°C. The reaction was terminated by the addition of 2 µl of 0.5 M EDTA. Ten µl of 80% glycerol/0.2% bromophenol blue were then added and the mixture was loaded on a 0.3 cm x 23 cm Biogel A-5m (Bio-Rad) column equilibrated with 1 mM Tris-HCl pH 8.0, 0.01 mM EDTA. One drop fractions were collected from the column, counted, and fractions containing the initial peak of radioactivity (excluding the two fractions just before the 'trough') were pooled in a siliconized tube and lyophilized overnight.

b) Poly-dG Tailing

Lyophilized cDNA was reconstituted in 72 µl of water, the radioactivity was counted, and the yield of first strand material calculated. This usually varied between 3 and 6 µg. Eight µl of sodium cacodylate (1.0 M, pH 7.0), 4 µl dGTP (20 mM), 1.6 µl $CoCl_2$ (100 mM), and 3 µl of terminal transferase (21U/µl, Ratliff Biochemicals, Los Alamos) were added to the cDNA and the mixture was incubated for 30 min at 37°C. A second 3 µl aliquot of enzyme was added and the incubation continued for an additional 30 min, followed by a third addition of enzyme (2 µl) and further incubation (total incubation time was 90 min at 37°C). To stop the reaction, 4 µl of 100 mM EDTA and 92 µl of TE buffer were added to the tube and the solution heated at 70°C for 5 min.

c) Second Strand cDNA Synthesis

To remove complementary mRNA and to prime the synthesis of the second DNA strand from the cDNA template, 0.8 µl of DNAse-free RNase A (1mg/ml, boiled 2 x 15

min, Boehringer-Mannheim) and 2.4 µl of oligo dC_{12-18} (1mg/ml, Collaborative Research) were added to the poly-dG tailed cDNA. The tube was heated in a boiling water bath for one minute and chilled on ice. 13.2 µl of 100 mM $MgCl_2$, 4.8 µl of all four dNTPs (5 mM each) and 4.0 µl of DNA polymerase I (200 U/µl, New England Biolabs) were added to the tube on ice and the reaction was carried out 21 hours at 14°C. The DNA polymerase was inactivated by the addition of 9.6 µl of 500 mM EDTA and heating at 70°C for 10 min.

d) Methylation

Internal Eco RI sites within the double-stranded cDNA (ds cDNA) were protected by treatment with Eco RI methylase. To achieve this, the tube containing the newly synthesized ds cDNA (see above) was chilled on ice and 5 µl of S-adenosyl-L-methionine (SAM, 1 mM, Pharmacia) and 2 µl of Eco RI methylase (40U/ml, New England Biolabs) were added. The methylation reaction was carried out at 37°C for 30 min, after which 200 µl of phenol-chloroform (1:1, equilibrated with TE) was added to the tube and the tube was vortexed and centrifuged. The aqueous phase was recovered and placed in a siliconized Eppendorf tube. The remaining organic phase was back extracted twice with 100 µl of TE and the TE phases were pooled with the original aqueous extract. The recovery of DNA into the aqueous phase was monitored by ^{32}P counts and was usually about 90%. The pooled aqueous phases were extracted three times with 800 µl of water saturated ether. Traces of ether were evaporated from the sample by heating to 65°C for 5 min and then by blowing nitrogen gas over the surface of the liquid for 30 min. The original aqueous volume (determined by marking the tube prior to heating) was restored by the addition of water as necessary. The DNA was precipitated by adding 15 µl of 100 mM spermine, vortexing, and leaving the tube on ice for 30 min. The DNA was recovered by centrifugation in an Eppendorf micro-centrifuge for 10 min and recovery was monitored by ^{32}P counts (usually >85%). One ml of 75%

ethanol, 0.3 M sodium acetate, 10 mM magnesium acetate was added to the DNA pellet and the sample was left on ice for one hour with occasional vortexing to remove the spermine. The DNA was recovered by centrifugation, the pellet was washed with 1 ml of 70% ethanol and stored on ice for 15 min. After the DNA was pelleted again, it was dried under vacuum for 5 min prior ligation to Eco RI linkers.

e) Eco RI Linker Ligation and Eco RI Cleavage

To prepare the phosphorylated Eco RI linkers, 6 µl of Eco RI linkers (1.0 A260 unit/100µl water, New England Biolabs), 6 µl of 0.5 M Tris-HCl pH 7.5/0.1 M MgCl2, 1 µl of ATP (100 mM), 3 µl of DTT (200 mM), and 44 µl of water were combined in a siliconized Eppendorf tube. 0.6 µl of T4 polynucleotide kinase (6 U/µl, Pharmacia) was added and the tube was incubated at 37°C for 20 min. An additional 0.6 µl aliquot of enzyme was added, and the tube incubated for a further 20 min, followed by a third aliquot and a third 20 minute incubation. The phosphorylated linkers were added on ice directly to the dried ds cDNA pellet (see above). 1.2 µl of ATP (100 mM) and 1.4 µl of T4 DNA ligase (400 U/µl, New England Biolabs) were then added to the tube and the contents were mixed thoroughly to ensure that the DNA pellet was dissolved. The ligation reaction was allow to proceed for 24 hours, after which the reaction was stopped by heating at 65 °C for 10 min and cooling on ice.

1.2 µl of 5 M NaCl and 2.3 µl of Eco RI (20U/µl, New England Biolabs) were added to the tube and the mixture was incubated at 37°C for a total of 90 min. Two aliquots of 2.3 µl Eco RI were added at 30 minute intervals during the reaction period. Eco RI enzyme was inactivated by heating at 65°C for 10 min and the tube was stored on ice prior to size selection by agarose gel electrophoresis.

f) Size Seleetion of cDNA

Ten µl of 10 x DNA loading dye (0.3 % bromophenol blue, 0.3% xylene cyanol and 40% glycerol) was added to the cDNA sample which was loaded and run on a

preparative 1.4% low melting point agarose gel (BRL). The gel was stained in 0.5 μg/ml ethidium bromide in water for 30 min, destained in water for 15 min, and photographed. The gel bands containing the cDNA of desired sizes were excised and transferred into 14 ml plastic tubes covered with tin foils. The gel was melted at 70°C for 10 min and then cooled to 37°C in a water bath.

The cDNA was recovered from the gel by the 'CETAB' method (Langridge et al., 1980). Two-phase 1-butanol solutions were prepared by first equilibrating the butanol with an equal volume of water. Hexadecyltrimethylammonium bromide (QN^+) (Sigma) was dissolved to 1% (w/v) in the butanol phase which was then shaken with an equal volume of the equilibrated aqueous phase. The two phases (butanol-QN^+ and H_2O-QN^+) were stored separately at room temperature and warmed to 37°C before use. For DNA extraction, an equal volume of the butanol-QN^+ and two volumes of the H_2O-QN^+ were added to the melted gel stored in the 37°C water bath. The tube was shaken vigorously for 5 min and centrifuged at 1000 g at room temperature for 2 min (with brake turned off). The butanol phase containing the cDNA was recovered, the aqueous phase was extracted twice with 1/2 volume of butanol-QN^+ and the butanol phases were pooled. The cDNA was recovered by extraction with 1/4 volume of 0.2 M NaCl and purified by extraction with phenol, chloroform, and finally precipitated with ethanol. The cDNA pellet was resuspended in 0.1 M KCl and further purified by precipitation with 6 μl of 100 mM spermine as described before. The DNA pellet was finally resuspended in 20 μl of water. The yield and the recovery of the cDNA during the purification was monitored by the ^{32}P counts, since the first strand of the cDNA was labeled.

g) Ligation of cDNA into Phage λgt11 Arms and *in vitro* Packaging

The cohesive Eco RI ends of phage λgt11 arms were reannealed by heating at 70°C for 5 min, cooled to 45°C for 30 min, and held on ice. 0.2 μg of a test insert (4 kb

ds DNA provided by Vector Cloning System) was ligated to 1.0 μg of phage arms as recommended by the protocol supplied with the kit. The reaction was carried out at 14°C for 2 to 24 hours in a buffer consisting of 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 20 mM DTT, .1 mM ATP, and 200 U (400 U/ml, New England Biolabs) in a volume of 5 μl. A control ligation containing water instead of DNA was always performed in parallel. The ligation reactions were terminated by chilling on ice and the material was processed for *in vitro* packaging.

The ligated phage DNA was packaged *in vitro* according to the protocol supplied with the Gigapack kit (Vector Cloning System). Ligated DNA (3 μl) was added to one tube of the Freeze/Thaw extract, followed by 15 μl of the Sonic extract. The tube was mixed well by stirring with a sterile plastic pipet tip. Care was taken not to let the temperature of the packaging extracts exceed 4°C. This was achieved by thawing the extracts and adding the reagents on ice. The packaging reaction was carried out at 22°C for 2 hours and was terminated by the addition of 0.5 ml of phage diluent and 20 μl of chloroform. After gentle mixing, the supernatant was diluted by consecutive double dilution, and the phage concentration titred in E coli. Y1088 at 42°C as described in Section F2, except for the fact that the phage were plated out on 90 mm Petri dish plates using 'top agar' that contained 40 μl of 40 mg/ml 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside (Xgal) (Pharmacia) and 40 μl of 1 M isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma) in order to allow the determination of the percentage of recombinant phage over the background (wild type β-galactosidase positive phage would produce blue plaques on these plates, while the recombinant β-galactosidase negative phage produced clear plaques).

A small fraction of the packaged phage was plated to determine the packaging efficiency. In addition, to ensure that the recombinant phage contained cDNA inserts and to determine the average insert sizes, 10 clear plaques were randomly picked with

Pasteur pipets and transferred to Eppendorf tubes containing 1 ml of phage diluent plus a few drops of chloroform. The phage in each tube were individually titred and the phage DNA isolated by the rapid method described in Section F2. Two µg of DNA from each sample was then cut with the restriction enzyme Eco RI (2 µl, 20U/µl, New England Biolabs) and analyzed by electrophoresis on 0.8% agarose gel.

h) Amplification of the cDNA Library

The packaged cDNA library was amplified in E. coli Y1088 before it was screened by antibody for cDNAs of interest. The phage were adsorbed to the E. coli Y1088 plating stock and plated on 20 large (150 mm) fresh LB plates (pH 7.5) at a density of 2 x 10^4 p.f.u.. The plates were incubated at 42°C for 6 to 8 hours until the plaques became confluent. They were then chilled to 4°C and 12 ml of phage diluent was added to each plate with a few drops of chloroform. The phage were eluted overnight at 4°C on a rotating platform with gentle agitation. The supernatant was collected from the plates and transferred to a sterile glass bottle. Five ml of chloroform were added and the phage stock was stored at 4°C. Each time the phage stock was used, 1 ml was removed from the bottle, diluted, and the phage titre determined accordingly.

I. ISOLATION OF POSITIVE CLONES FROM cDNA LIBRARIES

1. *Evaluation of Different Antibody Screening Methods*

The aim of screening a library with antibody was to isolate recombinant phage clones producing the protein of interest from a background population of thousands of plaques. A sensitive detection method was therefore essential for successful cloning. This was accomplished by using either Staphylococcus aureus ^{125}I labeled protein A, or a second antibody cross-linked to horseradish peroxidase (HRP) to detect the binding of the first antibody directed against the protein product expressed by the recombinant phage. Since no literature was available at the time this work was being carried out

comparing the advantages and disadvantages of each method, we wished to determine which method would be better suited for screening our libraries. A recombinant λgt11 phage stock, λSCS8 (which contained a cDNA insert encoding the partial sequence of rat liver α subunit of succinyl-Co A synthetase), and a rabbit polyclonal antibody recognizing the fusion protein produced by the phage stock were kindly provided by Dr. William Bridger, Department of Biochemistry, University of Alberta (Edmonton). The recombinant phage was mixed with an equal concentration of wild type λgt11 and the mixture was used to evaluate the two different methods.

One thousand p.f.u. of the phage mixture was plated on each 90 mm LB plate (pH 7.5) using fresh plating stock each time, as described before. Neither Xgal nor IPTG were added to the top agar since, at this stage of the experiment, the discrimination between recombinant and wild type phage was not necessary. The plates were incubated at 42°C for 4 to 5 hours until the plaques became visible (or reached the size of 1 mm in diameter). IPTG saturated nitrocellulose filters (Schleicher and Schuell, BA85) were prepared by soaking for 30 min in a 10 mM IPTG solution prepared in water and air dried for 1 hour. The IPTG-saturated filters were laid on top of the agar plates and the plates were transferred immediately to a 37°C incubator. Care was taken not to let the temperature of the plates drop below 37°C during the process. After incubation at 37°C for 2 to 3 hours to induce the production of fusion proteins, the plates were cooled to 4°C to harden the soft top agar. The position of each filter was marked asymmetrically in 5 to 6 places with a needle dipped in waterproof ink. The filters were then carefully removed (*i.e.* slowly peeled off the surface of the plate with forceps) and immersed in TBS buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl) immediately. All subsequent steps were carried out with gentle shaking on a rotating platform. The filters were rinsed in TBS for 5 min, "blocked" in 20% fetal calf serum in TBS (5 ml per 82 mm filter) at room temperature for 30 min, and exposed overnight at 4°C to 20% fetal calf serum in

TBS with a 1/200 dilution of the antibody recognizing the λSCS8 rat liver succinyl-Co A fusion protein (5 ml per 82 mm filter). Non-specific binding of first antibodies were removed by washing at room temperature once in TBS for 5 min, once in T-TBS (TBS plus 0.05% Tween-20) for 5 min and finally in TBS once again for 5 min. The filters were then immersed in 20% fetal calf serum in TBS containing ^{125}I-protein A from Staphylococcus aureus (1 x 10^6 cpm/82 mm filter, specific activity 30 mCi/mg, New England Nuclear) (5 ml per 82 mm filter) and incubated at room temperature for 2 hours. The filters were washed in TBS, T-TBS and TBS as before, dried and exposed to Kodak X-Omat AR film at -70°C.

Alternatively, the blocking buffer was replaced by 3% gelatin (from the Express-Blot kit, Bio-Rad) in TBS and the antibody and ^{125}I-protein A buffer was replaced by 1% gelatin in TBS. Furthermore, the secondary antibody, goat anti-rabbit IgG linked to HRP (from the Express-Blot kit, Bio-Rad) was also used to replace ^{125}I-protein A to detect the first antibody. Filters were rinsed, blocked (either with 20% fetal calf serum or 3% gelatin), incubated with the first antibody (in 20% fetal calf serum or 1% gelatin), and washed. They were then incubated with a 1/3000 dilution of the affinity purified and human IgG adsorbed HRP-conjugated goat anti-rabbit IgG (GAR-HRP, from the Express-Blot kit, Bio-Rad) in either 20% fetal calf serum/TBS or 1% gelatin/TBS, depending on what was used for the previous incubations. After sequential washes in TBS, T-TBS, and TBS (see above), the filters were developed either by using the HRP color development solution supplied with the Bio-Rad Express-Blot kit, or by employing a similar reagent, 3-amino-9-ethylcarbazole (Sigma). The latter was prepared fresh by dissolving 30 mg of 3-amino-9-ethylcarbazole powder in 7.5 ml of N,N-dimethyl formamide and adding the solution to 142.5 ml of 50 mM Na-Acetate pH 5.0 with 150 µl of H_2O_2. The reaction was stopped by rinsing in water. The filters were air

dried, sandwiched between Whatman 3 MM filter papers, wrapped in tin foil, and stored dark at room temperature.

2. *Reducing the Background Signals*

Phage plates were prepared, blotted, and processed as detailed above using a λgt11 cDNA expression library made from NALM-6 poly A^+ mRNA. The rabbit polyclonal antibody directed against denatured CD9 antigen (described previously in Section D) was used as the primary antibody for the screening regimen. The objective was to determine the optimal dilution of the first antibody required to obtain a weak, yet still visible background of antibody binding (see Chapter V for further discussion). To achieve this stock antiserum was diluted with antibody buffer (1% gelatin in TBS) to ratio of 1/100, 1/200, 1/400, 1/800, and 1/1600. Separate filters were incubated with each diluted antibody solution and processed according to the rinsing, GAR-HRP binding, washing, and developing schedule described above. The lowest antibody dilution that yield a barely visible background signal was chosen to perform the large-scale library screening.

Non-specific binding due to the cross reactivity of the rabbit polyclonal antibody to E. coli proteins was reduced by blocking the cross reactive antibodies with E. coli lysate. Systematically varied amounts of E. coli lysate (supplied as 1% solution; Bio-Rad) were added to appropriately diluted first antibody in 1% gelatin/TBS and the solution was rotated gently at 4°C for 30 min. This "adsorbed" first antibody solution was applied to filters washed and prepared as detailed above, using a small-scale phage preparation from the NALM-6 λgt11 library. The λSCS8 phage served as positive controls. From this series of experiments, the lowest concentration of the E. coli lysate that resulted in satisfactory background blocking was chosen for large-scale screening routine.

3. *Large-Scale Library Screening with Antibody Probe*

Fresh phage plating stocks were always used for large scale antibody screening. For this purpose, the E. coli strain Y1090 was streaked out on LB plates (pH 7.5) containing 50 μg/ml ampicillin and incubated at 37°C overnight to provide single colonies. From this culture, a single colony was isolated and grown overnight in 2 ml LB (pH 7.5) at 37°C. Twenty μl of the overnight culture were inoculated in 20 ml of LB (pH 7.5) supplemented with 0.2% maltose and incubated at 37°C to saturation (4 to 5 hours) with vigorous shaking. The bacteria were then resuspended in 1/2 volume of 10 mM $MgSO_4$ at a concentration of approximately 2.4 $O.D._{600}$ per ml. The plating stock (0.3 ml) was mixed with 0.1 ml of λ diluent containing 1 x 10^5 p.f.u. of the NALM-6 λgt11 cDNA library and the phage were allow to attach to the bacteria at room temperature for 20 min before they were plated out with 7 ml of the top agar (pH 7.5) on each 150 mm large plate (pH 7.5). Ten to twenty large plates were used each time in a typical screening. The phage incubation, the fusion protein induction, and the positive clone identification were carried out as described in the proceeding section. The phage were induced and later transferred to 132 mm nitrocellulose filters (Schleicher and Schuell BA85) which were processed in groups using separate 150 mm Petri dishes (two filters per dish, the sides of the filters with the bound proteins facing out). The desired clones were identified by gelatin blocking and GAR-HRP detection. Ten ml of the blocking and antibody buffers were used for each filter which was incubated with 1/400-fold diluted CD9 polyclonal antibody (optimal). The polyclonal antibody was adsorbed with a 1/200 dilution of E. coli lysate (0.005% final concentration) at room temperature for 30 min prior to use. The filters were then incubated with a 1/3000 dilution of the secondary antibody, GAR-HRP and the positive clones were visualized by developing in the color reagent.

In addition to screening the NALM-6 λgt11 library, a human hepatoma λgt11 cDNA library (kindly provided by Dr. William Bridger, Department of Biochemistry, University of Alberta, Edmonton) was also screened with the polyclonal antibody against the CD9 antigen under similar conditions.

Positive clones were isolated from the cognate agar plates using the fat end of a sterile Pasteur pipet. The agar plug was placed in 1 ml of λ diluent plus a few drops of chloroform, vortexed, and left at 4°C overnight. The phage stock was titered on 90 mm Petri dishes, and 1,000 p.f.u. of phage were plated out and subjected to a second round screening with the antibody. Positive plaques were picked from the culture plate using the sharp end of a Pasteur pipet. The agar plug was again eluted in 1 ml of λ diluent at 4°C overnight. This final phage stock was titred once more and a replicate nitrocellulose filter was lifted from a plate containing 50 to 100 well-isolated plaques. This filter was screened with the antibody for the last time (third round screening). If all the plaques gave positive signals, the phage stock derived from the second round of screening was then used for the isolation of phage DNA. If wild-type phage (identified as blue plaques) remained following the third round screening, further plating, screening, and plaque isolation were carried out until a homogeneous recombinant phage stock was obtained. The phage were finally stored in λ diluent over a few drops of chloroform at 4°C.

4. *Large-Scale Library Screening with cDNA Probe*

The NALM-6 λgt11 library was also screened with ^{32}P labeled DNA probes prepared from the positive clones identified from the human hepatoma λgt11 cDNA library by the rabbit polyclonal antibody directed against CD9 antigen. The library was reconstituted on a lawn of E. coli Y1090 plated on 150 mm LB plates (pH 7.5). The plates were incubated at 42°C for 3 to 4 hours until the plaques reached about 0.5 mm in diameter. The plates were then chilled in an upright position for 30 min at 4°C and dry nitrocellulose circles (*i.e.* which were not soaked in IPTG buffer as needed in antibody

screening) were applied to each plate for one minute (or until they became completely translucent, or wet throughout). After marking their orientation, the filters were lifted from the agar surface, soaked in denaturing buffer (1.5 M NaCl, 0.5 M NaOH) for about 45 sec, rinsed in neutralizing buffer (1.5 M NaCl, 0.5 M Tris-HCl pH 8.0) for a few sec, and soaked again in fresh neutralizing buffer for 2 to 3 min. A second, 'duplicate', filter was processed similarly. All filters were air dried and then baked at 80°C for 1.5 hours in a vacuum oven. The filters were prehybridized, hybridized to ^{32}P-labeled cDNA probe, and washed using the conditions and schedules described in Section G. Both sets of the filters (*i.e.* the original and its replicate) were hybridized to a common cDNA probe.

The washed nitrocellulose filters were air dried and taped to larger sheets of Whatman 3MM filter paper. The 3MM sheets were then labeled with several characteristic marks using ^{32}P-labeled ink and the nitrocellulose filters and the 3 MM papers were wrapped in Saran wrap and exposed to X-ray film. The labeled ink helped to align the developed film with the 3MM sheets and the position of the 'alignment holes' in the nitrocellulose filters could then be determined and marked on the film. In this way positive clones could be located on the original plate, and signals from duplicate filters could be aligned and compared. In general, clones were only considered truly positive if they gave a strong signal on both sets of the duplicate filters. The positive clones were picked and subjected to second and third rounds of screening as detailed in the proceeding section. Homogeneous recombinant phage stocks were isolated and stored as previously described.

J. CHARACTERIZATION OF CD9 cDNA CLONES

1. *Preparation of cDNA Inserts*

The positive plaques identified by the rabbit polyclonal antibody directed against the CD9 antigen were picked and stored as individual phage stocks. A particular phage stock was then used to infect a fresh E. coli Y1090 culture in order to isolate phage DNA by the rapid extraction method described in Section F. One to 2 μg of the DNA were digested to completion with 5 μl of Eco RI (20U/ml, New England Biolabs) at 37°C overnight in 50 to 100 μl volume of high salt buffer (100 mM NaCl, 50 mM Tris-HCl pH7.5, 10 mM $MgCl_2$, and 1 mM DTT). Five to 10 μl of 10 x DNA loading buffer supplemented with 1 mg/ml of RNase A (Pharmacia) were added to the digestion mixture and the cleaved DNA was analyzed by electrophoresis through a 0.8% agarose gel in the presence of 1.0 μg/ml of ethidium bromide. The gel was photographed and the size of the cDNA insert was estimated by plotting the position of the insert band against the DNA size markers run alongside the insert.

Alternatively, 1 to 2 μg of phage DNA was cleaved to completion with the restriction enzymes Kpn I (10-20 units/μl, New England Biolabs, Beverly, MA) and/or Sst I (4-6 units/μl, Bethesda Research Laboratories, Gaithersburg, MD) overnight at 37°C in 10 mM Tris-HCl pH7.5, 10 mM $MgCl_2$, and 1 mM DTT (low salt buffer). The digested DNA was then treated with RNase A and analyzed by agarose gel electrophoresis.

To prepare the insert for subcloning, 20 to 50 μg of the phage DNA were digested to completion with the enzymes Kpn I and Sst I overnight at 37°C in 300 μl of low salt buffer, treated with RNase A, separated on a preparative 0.8% agarose gel, after which the gel band containing the cDNA insert plus the flanking phage DNA (see chapter V for details) was excised, weighed, and placed into Eppendorf tubes. The DNA fragment was retrieved from the agarose matrix using the "Geneclean" kit (Bio 101, San Diego,

CA) according to the manufacturer's protocol. Briefly, 2 to 3 volumes of saturated NaI solution were added to the gel slice (0.2 ml of NaI per 0.1 g of gel) and the tube was incubated at 65°C for 3 min to dissolve the gel. Five to 10 µl of "glass milk" were then added, mixed, and the tube was set on ice for 5 min. The glass beads with DNA bound to their surface were recovered by centrifugation in an Eppendorf centrifuge for 2 sec. The pellet was then washed three times with ice-cold "NEW wash buffer", dried under vacuum, and resuspended in 10 to 20 µl of sterile water. The DNA was released from the glass beads by incubation at 55°C for 3 min and recovered by removing the beads through centrifugation. DNA purified by this method was used directly for subcloning.

2. *Subcloning into Plasmid and M13 Vectors*

pUC 19 plasmid DNA (Yanisch-Perron et al., 1985) (kindly provided by Dr. James Friesen, Department of Medical Genetics, University of Toronto, Toronto) was isolated from the host E. coli strain JM101 using the large-scale plasmid DNA extraction procedure detailed in Section F. Purified cDNA insert cleaved with Kpn I and Sst I was then ligated into Kpn I and Sst I cut pUC19 vector DNA dephosphorylated by treatment with calf intestinal phosphatase (Boehringer Mannheim) as described by Maniatis et al. (1982). Competent E. coli JM101 were prepared according to the procedure used to prepare competent E. coli JM103 (Davis et al., 1986), except that the 50 mM $CaCl_2$ solution was replaced with 10 mM $MgCl_2$, 5 mM Tris-HCl pH 7.5, and 100 mM $CaCl_2$. Two hundred µl of the competent cells were used for every 100 ng of plasmid vector (ligated in a volume less than 20 µl). Transformation of the competent cells by the recombinant plasmids was carried out on ice for 2 hours, after which the bacteria were seeded (without heat shock treatment) on 90 mm LB plates supplemented with 0.1 mg/ml of Xgal, 0.5 mM IPTG, and 50 µg/ml ampicillin. Colonies that grew on such plates either possessed recombinant plasmid (white colonies) or wild type pUC 19 (blue colonies).

Small-scale plasmid DNA preparations were obtained from isolated recombinant (*i.e.* white) colonies. The purified DNA was digested to completion with Eco RI enzyme, fractionated on a preparative 0.8% agarose gel, and the band containing the desired cDNA insert (Eco RI - Eco RI fragment) was excised and the DNA recovered by the Geneclean method.

The purified cDNA insert was then ligated into either M13mp 18 RF or M13mp 19 RF vectors (Norrander et al., 1983, Yanisch-Perron et al., 1985) (kindly provided by Dr. James Friesen) which had been cut with Eco RI and dephosphorylated. The preparation, transformation, and plating of E. coli JM101 cells were carried essentially as described above, except that the transformed bacteria were plated out along with 200 µl of indicator cells (log phase E. coli JM101) in 3 ml of top agar on Xgal-IPTG plates without ampicillin. After incubation at 37°C overnight, recombinant M13 bacteriophage would yield white plaques, while the wild type M13 phage gave rise to blue plaques.

The presence of cDNA inserts within the M13 vector DNA was confirmed indirectly by isolating M13 single stranded DNA. The white plaques were picked with the small end of a sterile Pasteur pipet and each agar plug was transferred into 2 ml of LB medium supplemented with 50 µl of log phase E. coli JM101. The cultures were allow to grow at 37°C for 4 to 6 hours with good aeration. One ml of the culture was then taken from each tube, and the bacteria were pelleted in an Eppendorf centrifuge for 5 sec. Ten µl of the supernatant (containing M13 recombinants) were removed, to which 10 µl of DNA loading buffer plus 1% SDS were added and the phage DNA was analyzed by 0.8% agarose gel electrophoresis. M13 vector containing cDNA inserts ran slower than the wild type M13 control.

Alternatively, if large numbers of recombinant phage plaques were to be tested for the presence of inserts, a simple and efficient phage culturing method was developed and used. White M13 phage plaques were picked and transferred into the wells of a

microtiter plate, each containing 100 µl of LB medium and log phase E. coli JM101. The plate was shaken for 4 to 6 hours on a microtiter shaker (MTS2, Terochem, Edmonton, Alta) which was placed in a bacterial incubator. The microplate was centrifuged in an IEC Centra - 7R^{TM} centrifuge at 3,000 r.p.m. for 10 min. Ten µl of the phage supernatant was removed from each well with a multichannel pipet and transferred to the well of another microtitre plate already containing 10 µl aliquots of DNA loading buffer plus SDS. After mixing with the multichannel pipet, the phage DNA samples were analyzed by gel electrophoresis.

3. *Generation of Deletion Mutants for Sequencing*

In order to facilitate the sequencing of the CD9 cDNA, the rapid method of M13 deletion subcloning which generated a nested series of sequencing templates was used (Dale et al., 1985). Briefly, 1 µl of M13-CD9 recombinant DNA (about 1.0 µg/µl), 1 µl of the RD20 (or Eco RI primer, 0.5 unit at O.D.$_{260}$ per ml, synthesized in Dr. James Friesen's laboratory), 2 µl of 10 x buffer (100 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$), and 14 µl of water were combined in an Eppendorf tube, incubated at 65°C for 10 min, and slowly cooled to room temperature over 30 min in order to anneal the primer to the single stranded M13 DNA. One µl of Eco RI (20 U/µl) plus 1 µl of water were added, and the tube was incubated at 37°C for one hour to cut open the double stranded region. Two µl of 10 x T4 DNA polymerase buffer, 2.5 µl of 100 mM DTT, and 1 µl of T4 DNA polymerase (0.5 to 2 U/µl. New England Biolabs) were then added, and progressively deleted insert fragments were generated at 37°C. Two µl fractions were recovered every 2 min and quickly heated to 65°C for 10 min in a water bath to inactivate the enzyme. After the sixth fraction, 2 µl samples were recovered every 3 min and the enzyme inactivated. All fractions were pooled into one Eppendorf tube to which 3 µl of 5 µM dGTP, 1.5 µl of water, and 1 µl of calf thymus terminal deoxynucleotidyl transferase (TdT, 4U/µl, Pharmacia) were added, and the dG-tailing reaction was carried

out at 37°C for 20 min and stopped by heating at 65°C for 10 min. One µl of the Eco RI primer was again added and reannealed to the cleaved single stranded DNA. The nick was sealed by the addition of 3 µl of ATP (10 mM), 4 µl of water, and 1 µl of T4 DNA ligase (100 U/µl, New England Biolabs). The ligation reaction was carried out at room temperature for at least one hour. The ligated deletion mutants were then used to transform E. coli JM101 competent cells as detailed above. DNAs isolated from the white plaques were analyzed by agarose gel electrophoresis in order to confirm the presence of deleted inserts.

Alternatively, the CD9 cDNA insert was subcloned in vector M13mp10 RF (from Dr. Friesen's laboratory). The replicative form of the recombinant M13 DNA was then isolated by CsCl banding (see Section F2), and subjected to deletion by Exonuclease III and VII (Yanisch-Perron, et al., 1984). Briefly, the recombinant RF M13 DNA was co-digested with Bam HI and Pst I restriction enzymes, cleaned, and the insert was progressively digested in the 3' to 5' direction with Exonuclease III (New England Biolabs). Overhanging single stranded DNA was then trimmed away with Exonuclease VII (Bethesda Research Laboratories). Blunt ends were created using the large fragment of DNA polymerase I (Bio-Rad), and ligated. The deletion mutants were selected and analyzed as detailed above.

4. *Sequence Analysis of the CD9 cDNA*

Single stranded M13mp18 containing the entire CD9 cDNA insert was used to obtain the CD9 nucleotide sequence by the dideoxynucleotide chain termination method developed by Sanger et al. (1977).

Since the cDNA was subcloned into the unique Eco RI site of the M13mp18, it was necessary to determine the orientation of the insert in relation to the priming site for sequencing. This was achieved by the C-test described by Messing (1983), using phage supernatants prepared by the rapid method discussed in Section F. Ten µl of phage

supernatant were added to 10 µl of 5 to 10 randomly selected phage supernatants with 1 µl of 2% SDS. The tubes were heated at 65°C for 1 hour and cooled to room temperature for 10 min in order to anneal DNA sequences. The solutions were then mixed with DNA loading buffer and analyzed by agarose gel electrophoresis. If two annealing inserts were indeed in opposite orientation, they would be expected to reanneal to each other such that they migrate to higher molecular weight regions on the gel. On the other hand, inserts in identical orientation could not reanneal. These would remain single stranded and band at lower molecular weight regions on the gel.

a) Purification of Sequencing Templates

White plaques representing recombinant M13mp19-CD9 clones were picked with the sharp end of Pasteur pipets and each agar plug was inoculated into 2 ml of LB medium in a glass tube containing 20 µl of a fresh overnight culture of E. coli JM101. The inoculates were allow to grow for 6 hours at 37°C with vigorous shaking. 1.5 ml of the resulting culture were then removed and transferred to individual Eppendorf tubes. The bacteria were pelleted in an Eppendorf micro-centrifuge for 5 min and 1 ml of the supernatant was carefully retrieved. The pellet was stored at 4°C in the event that it could be cultured once again in the future, if necessary. 250 µl of 20% polyethylene glycol (PEG)/2.5 M NaCl were added to the supernatant, mixed, and left to stand at room temperature for 30 min. The phage particles were recovered by centrifugation in an Eppendorf micro-centrifuge for 5 min. The supernatant was discarded and the walls of the tube were wiped with Kleenex tissue to remove traces of PEG. The phage particles were then resuspended in 100 µl of TE buffer and the phage DNA was extracted with 100 µl of phenol by repeated vortexing and the tube was allowed to stand at room temperature for 5 min. The phases were separated by centrifugation in an Eppendorf micro-centrifuge for 4 min and no more than 80 µl of the aqueous phase was removed (to avoid picking up any protein from the interphase). The sample was extracted twice

with an equal volume of ether, after which 1/10 volume of 3 M NaOAc pH 4.8 were added and the ssDNA was precipitated with 200 µl of 95% ethanol at -70°C for 30 min. The DNA was pelleted for 10 min at 4°C in an Eppendorf micro-centrifuge, washed once with ice-cold 70% ethanol, pelleted again, dried briefly under vacuum, and resuspended in 50 µl of TES buffer (20 mM Tris-HCl pH 8.0, 10 mMNaCl, 0.1 mM EDTA). Five µl of ssDNA templates prepared in this fashion were analyzed by 0.8% agarose gel to verify the purity and to estimate the yield.

b) Sequencing Reactions

To anneal the sequencing primer, 8 µl of ssDNA template (prepared as described above), 1 µl of the 17-mer primer (0.03 $O.D._{260}$ per ml, New England Biolabs), and 1 µl of A/R buffer (100 mM Tris-HCl pH 8.0, 100 mM $MgCl_2$) were combined in an Eppendorf tube, incubated at 65 °C for 5 min, and cooled for 10 min at room temperature. The liquid condensate on the tube walls was collected by a short pulse centrifugation in an Eppendorf micro-centrifuge. One µl of 100 mM DTT, 2 µl of α-^{35}S-dATP (>1000Ci/mmol, New England Nuclear) and 1 µl of DNA polymerase I Klenow fragment (3 to 8 U/µl, New England Biolabs) were added. From this "primed template" mixture, 3 µl were added to each of four Eppendorf tubes containing 2 µl of mix A, mix G, mix C, mix T (prepared as summarized in Table 2). Sequencing reactions were initiated at 37 °C for 15 min followed by the addition of 1 µl of "chase" solution (0.125 mM of the four dNTP) for another 15 min at the same temperature. The reaction was stopped by adding 6 µl of sequencing dye (80% formamide, 0.1% bromphenol blue, 0.1% xylene cyanol in 1 x TBE) to each tube. The samples were then either boiled for 5 min and loaded immediately on sequencing gels, or stored at -20°C (without boiling) until used.

TABLE 2

PREPARATION OF DIDEOXY MIXES FOR DNA SEQUENCING

1. Deoxy NTP mixes (dATP*, dGTP*, dCTP*, and dTTP*)

	dATP*	dGTP*	dCTP*	dTTP*
0.5 mM dGTP	20 µl	1.0 µl	20 µl	20 µl
0.5 mM dCTP	20 µl	20 µl	1.0 µl	20 µl
0.5 mM dTTP	20 µl	20 µl	20 µl	1.0 µl
TE buffer	20 µl	20 µl	20 µl	20 µl

2. Dideoxy mixes (mix A, mix G, mix C, and mix T)

	mix A	mix G	mix C	mix T
30 µl deoxy mixes	dATP*	dGTP*	dCTP*	dTTP*
30 µl ddNTPs†	0.1mM ddATP	0.3mMddGTP	0.1mM ddCTP	0.5mM dTTP
3 µl dATP	0.01mM dATP	0.01mM dATP	0.01mM dATP	0.01mM dATP

§All the dNTPs were purchased from Pharmacia

†All the ddNTPs were purchased from BRL

The mixes were stored at -20°C for up to one month

c) Sequencing Gels

Polyacrylamide sequencing gels (8 M urea, 0.4 mm thick) were prepared essentially as described by Davis et al. (1986). The glass plates (34 x 30 cm; BRL) used to cast the gels were thoroughly cleansed by brushing with Ajax detergent in hot running water, rinsed with hot water, washed in 1 M KOH/95% ethanol, rinsed with 95% ethanol and air dried before being assembled. A stock solution of 5% acrylamide and 8 M urea in 1 x TBE buffer was filtered and degased prior to use. Two shark's tooth combs (BRL) were used to form a total of 64 separate wells per each 34 cm gel (each well measures 6.4 mm wide) thus allowing a total of 16 sequencing lanes to be run on a single gel. The casted gel was assembled on a BRL Model SO sequencing apparatus. TBE buffer (1 x) was used to fill the upper and lower electrode chambers. A 20 x 40 cm aluminum sheet was placed in contact with the anterior glass plate to ensure adequate heat exchange and eliminate the "smiling" effect. Each gel was pre-electrophoresed at 1,300 volts for 30 min prior to loading. Three µl of heat-denatured sample were loaded into each well using a 10 µl Hamilton syringe fitted with a 26 gauge needle. The gel was run at 40 watts constant power for a total of 3 to 4 hours until the bromophenol blue dye marker reached the bottom of the gel. When required, multiple loadings were performed when the dye front of the previous loading had reached the bottom of the gel. Following electrophoresis, the gels were fixed in 10% methanol, 10% acetic acid for 15 min at room temperature in a large tray, covered with Saran wrap, and dried under vacuum for 1 to 2 hours at 80°C in a Bio-Rad gel dryer. The Saran wrap was then removed, and the gels were exposed overnight to Kodak XAR-5 films at room temperature. DNA sequences were read and recorded manually. The sequence data was stored and analyzed using the University of Wisconsin DNA analysis program and a Vax computer. DNA

and protein sequences comparisons were performed by searching the Genebank and Bionet database.

d, Synthesis of Sequence-Specific Primers

DNA sequence information obtained from sequencing both strands of the CD9 insert from the universal primer were used to design two 17-mer primers specific for CD9 cDNA, namely ori 1A (orientation 1A : 5'CACTTGCTGATAGGGTC3') and ori 2A (orientation 2A: 5'GGGAATTACTCAGGAGC3'). Two other 17-mer primers (ori 1B: 5'CTTTGGGGTGATTGAAG3'; and ori 2B: 5'GGTGGCTTGTCTTGAGG3') were also constructed using the sequence information derived from sequencing off the ori 1A and ori 2A specific primers. These oligonucleotides were synthesized in Dr. James Friesen's laboratory (Department of Medical Genetics, University of Toronto, Toronto) on an Applied Biosystems Model 380B DNA synthesizer using phosphoramidite chemistry. Once synthesized, the oligonucleotides were cleaved from the polystyrene support and were purified according to the protocol used in the laboratory. Briefly, the base protecting groups were removed by treatment with 1 to 2 ml of NH_4OH at 55°C overnight in a sealed ampoule. The solution was then cooled to room temperature, dried under vacuum, and dissolved in 0.3 ml of water in order to measure the O.D.260. Ten O.D.260 units of each oligonucleotide were then dried once more under vacuum and dissolved in 90% formamide, 10 mM Tris-HCl pH 8.0, and 1 mM EDTA. The oligonucleotide was denatured at 65°C for 10 min, quickly chilled on ice, and separated on a preparative 7 M urea, 20% polyacrylamide gel. The DNA was visualized over a silica gel GF UV-sensitive thin layer chromatography plate (Fisher) and the band containing the desired oligonucleotide was excised from the gel and crushed. The DNA was eluted overnight at 37°C in 5 ml 0.5 M NH_4OAc and filtered through a C_{18} column (Sep-Pak C_{18} cartridges #51910, Millipore Water Associates). The column was first washed with 10 ml of acetonitrile followed by 20 ml of water and

the DNA eluted with 2 ml of 60% methanol in four 0.5 ml fractions. The solution was dried under vacuum before being resuspended in 0.2 ml of water and the concentration determined by measuring optical density at 260 nm.

e) Sequencing with Double-Stranded DNA Templates

Double-stranded DNA fragments containing CD9 insert were recovered from preparative agarose gels by electroelution. The DNA was extracted twice with phenol and ether before being precipitated with ethanol. The DNA was resuspended in TE buffer and denatured by treatment with alkali (Zhang et al., 1988). Briefly, 2 µl of 2 N NaOH and 2 mM EDTA were added to 20 µl of the ds DNA (1 µg) and the mixture was heated at 65°C for 5 min. The solution was neutralized by addition of 2 µl of 2 M ammonium acetate, pH 4.5 and the denatured DNA templates were precipitated with ethanol. The DNA pellet was dried under vacuum, resuspended in 10 µl of sterile water, and a 10-fold excess of the λgt11 primer (New England Biolab) was added together with 1/10 volume of the A/R sequencing buffer. The solution was heated to 37°C for 15 min in order to anneal the primer. The "primed" DNA was then used to perform the dideoxy chain termination sequencing reaction as described above.

5. *Expression of CD9 cDNA in NIH 3T3 Cells*

a) Subcloning into Expression Vector

The expression vector pcEXV-3 (obtained from J. Miller, NIH, Bethesda) contains the SV40 early promoter in an orientation enabling it to drive the transcription of the cDNA sequence inserted downstream. In addition, SV40 poly adenylation signals are also present downstream from the cDNA insertion site (Miller and Germain, 1986). The CD9 cDNA could be cloned into the unique Eco RI site present in the cDNA insertion site. The plasmid DNA was therefore isolated by the Triton-CsCl method described in Section F1, cut with Eco RI, and dephosphorylated with calf intestinal phosphatase. Purified CD9 cDNA insert was then ligated into the Eco RI site of the

pcEXV-3 vector. The orientations of the cDNA inserts were determined by mapping with the restriction endonucleases Nru I and Hind III. Recombinant plasmids with CD9 cDNA in both orientations were obtained. Plasmids with the insert in the 'anti-sense' orientation would serve as negative controls for transfection and expression. Large-scale plasmid DNA preparation was carried out using the Triton-CsCl method in order to obtain pure closed circular DNA (cccDNA). The purity of the cccDNA was verified by 0.8% agarose gel electrophoresis.

b) Expression of CD9 cDNA by Polybrene-Assisted Transfection

Transfection of NIH 3T3 cells was accomplished by the polybrene-assisted gene transfer method developed by Aubin et al., (1988). Briefly, log phase NIH 3T3 cells were seeded at 2.5 to 5.0 x 10^5 cells/ 60 mm dish and allowed to grow for 18 hours at 37°C in tissue culture incubator. The culture medium was then removed and the cell monolayers were gently overlaid with 2 ml of 'transfection mix' which consisted of 10 µl of 1 mg/ml polybrene (Aldrich Chemical Company; prepared in Hank's Balanced Salt Solution) and 20 µl of 1 ng/µl pcEXV-3/CD9 DNA in complete growth medium. The polybrene-DNA-protein complexes were allowed to adsorb to the cells for 20 hours at 37 °C in the tissue culture incubator, after which the 'transfection mix' was gently aspirated. The monolayers were then carefully overlaid with 4.5 ml of freshly prepared, pre-warmed, complete growth medium containing 15% (v/v) dimethyl sulfoxide (DMSO, Fisher). Following incubation at 37°C for 5 min, the DMSO-containing medium was removed and the monolayers were washed twice with warm growth medium. Five ml of growth medium containing 10 mM sodium butyrate (Sigma Chemical Company) were then added to each monolayer and the dishes were again incubated at 37°C in tissue culture incubator before the monolayers were harvested 3 days after the transfection. After washing twice with PBS, the cells were dislodged from the bottom of the dishes by treatment with trypsin, pooled, washed once more with PBS

and were analyzed for CD9 protein expression either by Western blotting and/or by indirect immunofluorescence microscopy.

CHAPTER III

PURIFICATION OF CD9 ANTIGEN AND PRODUCTION OF POLYCLONAL ANTIBODY

A. INTRODUCTION

Following the initial immunobiochemical studies of CD9 antigen with the monoclonal antibodies, isolation of sufficient amount of purified CD9 protein became one of the major priorities for the characterization of CD9 structure and function. Gene cloning necessitates selection of the correct clones either by an antibody of high specificity, or by the use of oligonucleotide probes. Since the peptide derived amino acid sequence of CD9 glycoprotein is not available, purification of large amounts of CD9 antigen was an absolute necessity in order 1) To raise polyclonal antisera against denatured CD9 antigen which would serve as a probe to identify the CD9 producing clones from a cDNA expression library, and 2) To obtain sequence information for the generation of oligonucleotide probes which could then be used to clone the CD9 cDNA.

As mentioned in the first Chapter, the emergence of monoclonal antibodies in the past decade has greatly facilitated the identification, purification, and characterization of specific plasma membrane proteins. The unique resolving power of the monoclonal antibodies in detecting individual components has made it possible to purify a large number of membrane proteins through antibody affinity chromatography. The technique involves immobilizing the antibody on a solid support matrix, which was used to selectively adsorb the corresponding antigen from a mixture containing many proteins (Livingston, 1974; Dalchau and Fabre, 1982). The proteins that did not bind to the antibody were washed away, and the purified antigen then separated and eluted from the matrix. The monoclonal antibody affinity chromatography provided the opportunity to isolate proteins according to their specific interaction with the antibody, and thus differed

radically from the conventional chromatographic techniques in which separation depended on gross physical and chemical differences between the substances.

There are a variety of commercially available matrices to which monoclonal antibodies may be covalently coupled (Jakoby and Wilchek, 1974; Pharmacia technical brochure, 1977), these matrices differing in the reactive groups used in the linkage. Non-covalently coupled matrices were also used in the past, but due to the antibody loss during the washing and during the elution, they were largely replaced by the former type of matrices. Among the the matrices used for antibody cross-linking, cyanogen bromide-activated Sepharose was probably the most commonly used. Antibody was covalently coupled to the CNBr-activated Sepharose via primary amino groups. The coupling procedure was simple, and consisted essentially of mixing the matrix and the antibody at the correct pH. The use of this matrix, however, often generated affinity columns with low antibody activity due to the multisite attachment and improper orientation of the immunoglobulin molecule, which reduced the efficiency of antibody/antigen interaction. Although the addition of a six-carbon long spacer group to the Sepharose (Sepharose 4B) improved the antibody activity, the problem of the poor antibody orientation still remained. The ideal solution would be to somehow link the Fc region of the antibody to a solid support, or even better, through a flexible spacer such as the six-carbon spacer used in Sepharose 4B, since this would enable the Fab regions to more efficiently bind antigen. Schneider et al. (1982) developed a one-step membrane protein purification method that appeared to meet this requirement. In this method, the antibody was optimally oriented by cross-linking through dimethyl pimelimidate to protein A-Sepharose CL-4B. The affinity matrices were stable to high and low pH buffers without any significant antibody loss. The high efficiency of antigen binding exhibited by the antibody affinity column permitted the purification of several membrane proteins to be isolated, including the HLA-AB antigen, the transferrin receptor, the common acute

lymphoblastic leukemia antigen (CALLA) (Schneider et al., 1982). Coincidentally, the cell line used in the study of HLA-AB and CALLA purification was the NALM-6, the same cell line intended for CD9 antigen isolation. Because of the high efficiency and the simplicity of the procedure, this method was chosen to carry out the CD9 antigen purification, although several modifications were made to improve the protein recovery. The purified CD9 antigen would then be used to immunize animals in order to raise polyclonal antibody against the denatured CD9 protein.

One of the most important differences between the monoclonal and the polyclonal antibodies is the number of epitopes recognized by each antibody. Although the unique attribute of monoclonal antibody to recognize one epitope on a complex antigenic structure makes it a powerful tool for biological analysis of complex structures, its dependence on a single epitope would limit its application in certain circumstances. For example, the monoclonal antibody would fail to recognize a specific antigen if the region that carries the epitope is missing from the molecule, a situation frequently encountered in gene cloning, when the cDNA encoding the antigen is expressed. Furthermore, if the epitope recognized by the monoclonal antibody is conformationally determined by a disulfide bond, the antigen would no longer be detected once reduced. Polyclonal antibody would still be expected to recognizing the antigen in such situations.

Protocols for animal immunization vary considerably between investigators and depend to a large extent on the quantities and the type of antigens used. The immunization schedule basically consists of the priming injection followed by several boosting injections with 10 to 20 days in between each injection. To elicit a strong immune response, adjuvants are usually employed during immunization. Complete Freund's adjuvant is the most commonly used adjuvant, which contains heat-killed bacteria to stimulate the animal's immune system, with an oil base in which the immunogen is emulsified to ensure slow release. Incomplete Freund's adjuvant only

contains the oil base, with no bacteria in it. If the antigen used has a small molecular weight, such as synthetic peptide, carrier proteins are routinely used to conjugate to the antigen in order to elicit an adequate immune response. In most animal immunizations, pure antigen or immunoprecipitates were used as immunogens. However, if the pure antigen is difficult to isolate, gel strips containing the protein have been successfully used to raise the antisera, as in the case of Simian Virus 40 T Antigen (Carroll et al., 1978).

B. RESULTS

1. *NALM-6 Cell Lysate Preparation*

To prepare a cell lysate for large scale CD9 antigen isolation, 1×10^{11} NALM-6 cells were grown up in 250 large tissue culture flasks with 200 ml 10% RPMI medium per flask, and harvested when the cell concentration reached 2×10^{6} cell/ml. The cell viability of each batch culture was determined by Trypan blue dye exclusion test, and less than 5% of cell tested each time took up the dye. The cells were lysed with 300 ml of 20 mM Tris-HCl pH 8.0, 1 mM Na_2HPO_4, 0.137 M NaCl and 0.5% NP-40, and the non-specific binding proteins were removed by adsorption with the 6 g of PBS washed Pansorbin as outlined in Materials and Methods.

2. *Coupling of mAb 50H.19 to Protein A-Sepharose CL-4B*

The mAb 50H.19 partially purified by ammonium sulfate was cross-linked to protein A-Sepharose CL-4B. 15 ml of the mAb 50H.19 (9 mg/ml, 135 g total) was used to couple to 3.15 ml of the packed beads in 40 ml of borate buffer for 30 minutes. After removing the antibody bound Sepharose, the supernatant was analyzed for the presence of the unbound antibody. 10 µl of the supernatant as well as a control of 10 µl antibody solution containing an equivalent amount of unabsorbed mAb 50H.19 were mixed with the protein sample buffer, denatured and assayed by a 12.5% SDS-PAGE, followed by

silver staining. The absence of the immunoglobulin in the supernatant was determined by the disappearance of the 25 kDa band (light chain) and the 50 kDa band (heavy chain) respectively, compared to the strong immunoglobulin bands in the control. In addition, the presence of the mAb 50H.19 on the Sepharose beads was tested by immunoprecipitating of 1 x 10^6 ^{125}I-labeled NALM-6 cell lysate with 30 µl of the antibody cross-linked beads (about 0.01 g dried Sepharose). The CD9 antigen precipitated by the antibody cross-linked beads was visualized by fluorography.

3. *Modification of Purification Procedure*

For initial CD9 purification, NALM-6 lysate prepared from $1x10^{11}$ cells as described above was incubated with 4.7 ml of the antibody-crosslinked Sepharose, packed into an 8 ml econo-column, washed, and eluted in 1 ml fraction into 40 Eppendorf tubes, each containing 0.1 ml of 0.5 M NaH_2PO_4 as described in the previous chapter. 20 µl of the column eluate from each tube was analyzed for the presence of the CD9 antigen by SDS-PAGE, followed by silver staining. No CD9 protein band, however, could be detected although BSA controls (0.2 µg, 0.5 µg, and 1 µg) were clearly evident.

In order to detect the CD9 antigen in the column eluate, the 40 fractions were lyophilized. However, after the volume was reduced to 2 ml, the eluate became very viscous, presumably due to the concentrated deoxycholate. In addition, bicarbonate salt began to crystallize, because of oversaturation. This made the solution very difficult to handle.

To separate the deoxycholate and the bicarbonate, the concentrated column eluate was diluted with an equal volume of water, and dialyzed extensively against water or 0.001 M Tris-HCl pH 7.2 buffer. Although most of the bicarbonate was removed as evidenced by the disappearance of the salt crystals, the eluate still remained viscous. The eluate was again diluted with 2 volumes of the Tris buffer (12 ml total) and pressure

dialyzed with an ultra-filtration apparatus. Deoxycholate still remained after the dialysis, however, judging by the viscosity of the eluate after concentration.

To solve the problem caused by deoxycholate, 5×10^7 NAML-6 cells were surface labeled with ^{125}I, and after extensive washing in PBS, the labeled cells were lysed in 0.6 ml of 20 mM Tris-HCl pH 8.0, 1 mM Na_2HPO_4, 0.137 M NaCl and 0.5% NP-40. 3×10^6 cpm (380 µl) of the lysate was coupled to 1 ml of the freshly made mAb 50H.19-protein A-Sepharose. The coupling, the washing, and the elution were carried out as before, except that the column was first eluted with the solution that did not contain the 0.5% deoxycholate, and the eluate was collected with Eppendorf tubes containing 1/10 volume of 2% acetic acid [determined by titering to neutral pH the 0.05 M diethylamine (pH 11.5) with 1/10 volume of various concentration of acetic acid]. That the radioactivity in the column eluate represented CD9 antigen was confirmed by SDS-PAGE, followed by fluorography. The proportion of proteins removed during washing, eluted from the column in the absence and presence of deoxycholate, and remaining after elution was determined by measuring their radioactivity, and is presented in Table 3. Proteins non-specifically bound to the column (61%) were removed in the washing fraction. Elution with the deoxycholate-free solution recovered 6.7% proteins (CD9-specific). Further elution with the solution containing 5% deoxycholate recovered an additional 2.8% proteins. However, 11.7% proteins still remained tightly bound to the column. Although more stringent elution condition may dissociate these proteins from the column, this probably would result in antibody loss. On the other hand, removal of 0.5% deoxycholate from the elution solution only resulted in a drop of 20% in antigen recovery from the affinity column. No salt crystal was formed after concentrating the deoxycholate free column eluate either. Therefore, in the subsequent CD9 purification, the affinity column was eluted with the deoxycholate-free solution, and the column eluate was neutralized with 1/10 volume of 2% acetic acid.

TABLE 3

COMPARISON OF THE COLUMN ELUTION WITH AND WITHOUT DEOXYCHOLATE

	with Deoxycholate (cpm x 10^6)	recovery %	without Deoxycholate (cpm x 10^6)	recovery %
Total Lysate	3.000	100	3.000	100
Washing	1.830	61	1.830	61
Eluate	0.284	9.5	0.200	6.7
Column After Elution	0.350	11.7	0.434	14.5

NALM-6 cell were labeled with ^{125}I and lysed.

3 x 10^6 c.p.m. of labeled surface proteins were loaded on a mAb 50H.19-protein A-Sepharose CL-4B column. The bound antigen was eluted in the presence or absence of the 0.5% deoxycholate. Most CD9 mAb non-specific proteins were washed away (61%) before the elution. Significant amount of proteins (11.7%) remained even after elution with the solution containing 5% deoxycholate. Removal of deoxycholate only caused a 2.8% drop of the eluted CD9 antigen.

4. *Overcoming Non-specific Binding of CD9 Antigen*

While experimenting with different elution conditions the column purified ^{125}I-labeled CD9 antigen was bound to the freshly made affinity matrix. However, it was noticed that much less antigen could be recovered once it was passed through another affinity chromatographic procedure, even when conditions identical to the original purification were used. Careful tracing of the radioactivity during chromatography revealed that the CD9 antigen would bind non-specifically to 'everything', including the Eppendorf tube, econo-column, Sepharose beads, and pipet tips.

To avoid the protein loss due to non-specific adsorption by CD9 antigen, all the tubes, and tips used were first 'coated' with BSA by soaking in 1 mg/ml BSA in PBS for 30 minutes at room temperature followed by extensive washing with PBS. The affinity matrix was also treated with the same BSA and washed before the antigen coupling and elution. Although the BSA treatment greatly reduced the non-specific binding of CD9 antigen, BSA was found in the CD9 column eluate, as determined by silver staining. Reverse phase HPLC was used to separate the BSA from the CD9 protein. Unfortunately, the BSA co-migrated with the CD9 protein under the condition used (as evidenced by the overlap between the peak of the BSA in HPLC and the peak of CD9 radioactivity), and could not be separated. 1% SDS was then added to the BSA-CD9 solution to a final concentration of 0.1% in an attempt to dissociate the BSA and CD9. All three components co-migrated on the reverse phase HPLC, and could still not be separated (result not shown).

An interesting finding was that when "old tubes" (the tubes that contained NALM-6 lysate before) were used, much less non-specific binding of the ^{125}I-labeled CD9 to the tubes occurred. Therefore all laboratory ware such as Eppendorf tubes, centrifuge tubes, econo-columns, and even pipet tips used in the subsequent CD9 antigen

purification were pre-incubated with the CD9 containing cell lysate, and stored at -20°C for the next batch of purification.

5. CD9 Antigen Purification from NALM-6 Cells

With the improved elution protocol, and having solved the antigen loss problem, 2 x 10^{11} NALM-6 cells were again grown in culture and used for CD9 protein isolation. 100 ml of the clarified, Pansorbin adsorbed lysate was bound to 7 ml of the mAb 50H.19-protein A-Sepharose CL-4B for each batch of purification, and the column reused after reconstitution with alternating high and low pH treatment. The column was eluted in the absence of deoxycholate, and the eluate was collected in 1 ml fraction into the tube containing 1/10 volume of 2% acetic acid. The volume of each fraction was reduced 10 fold by lyophilization. 10 µl was taken from each fraction, and analyzed for the presence of CD9 antigen by SDS-PAGE, followed by silver staining. Figure 2 shows the staining result. CD9 was detected in fraction # 5 and #6. The protein concentration in each lane was estimated by the control BSA. Compared to lane b (1 µg BSA), there was about twice as much protein in lane e (2 µg CD9 protein per 10 µl, or 200 µg/ml), and three times as much in lane f (300 µg/ml). In addition to the CD9 protein bands (the major 22 kDa and the minor 24 kDa), another two protein bands were detectable in the NALM-6 column eluate, a 45 kDa major band and a 48 kDa minor band. The mAb 50H.19 affinity chromatography using NALM-6 lysate was repeated after reconstituting the column, and 1.4 mg of the column purified CD9 antigen was recovered from the entire lysate. Figure 3 shows a summary of the modified mAb 50H.19 affinity chromatography procedure.

6. CD9 Antigen Purification from Platelets

Since CD9 antigen was also expressed on platelets, and out-dated human platelets were sometimes available from the local Red Cross Center, the possibility of using the platelets to replace NALM-6 for antigen isolation was explored. 20 ml of peripheral

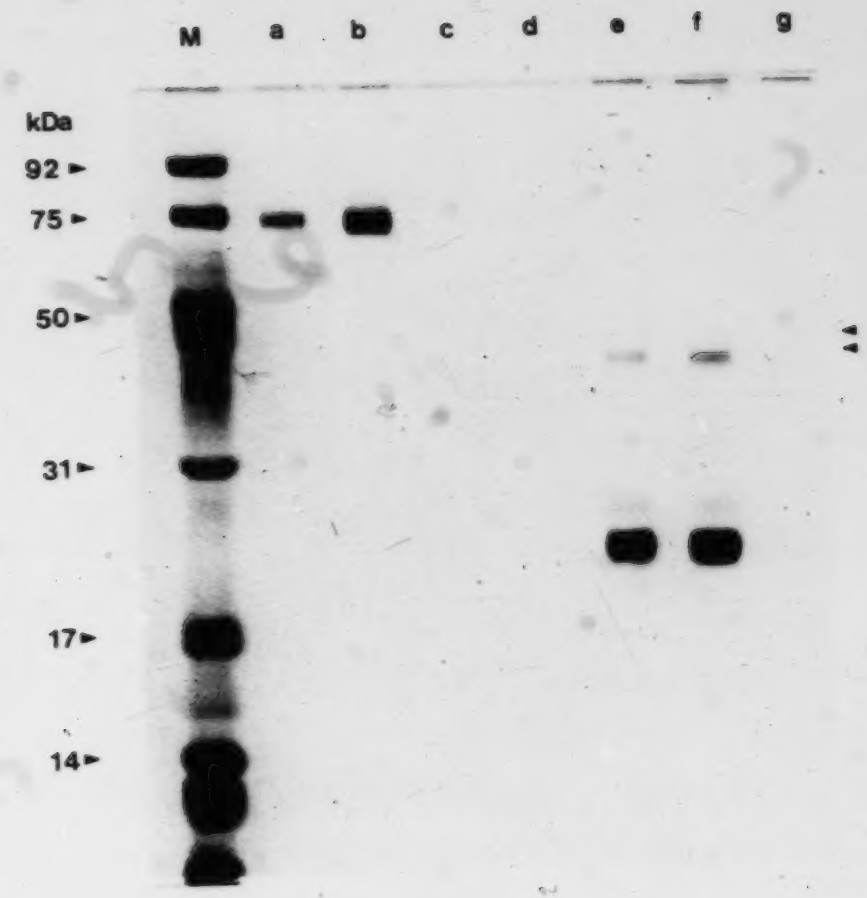


FIGURE 2. Analysis of the CD9 antigen purified from NALM-6 cells by SDS-PAGE. 10 µl of the concentrated column eluate from each fraction was mixed with 10 µl of the sample buffer, and analyzed by SDS-PAGE using a 12.5% acrylamide gel. The gel was silver stained and the quantity of CD9 protein estimated by comparing to the BSA protein standard. Lane M is the Bio-Rad low molecular weight protein markers. Lane a and lane b were loaded with 0.5 µg and 1.0 µg of BSA respectively. Lane c to lane g were loaded with samples from fraction #3 to fraction #7 of the mAb 50H.19 affinity column eluate. Two additional minor protein bands present in lane e and f, had molecular weights of 45 kDa, and 48 kDa respectively (arrowed). The ratio of the intensity of the 45 kDa to 48 kDa kilodalton bands is very similar to that of gp22 to gp24.

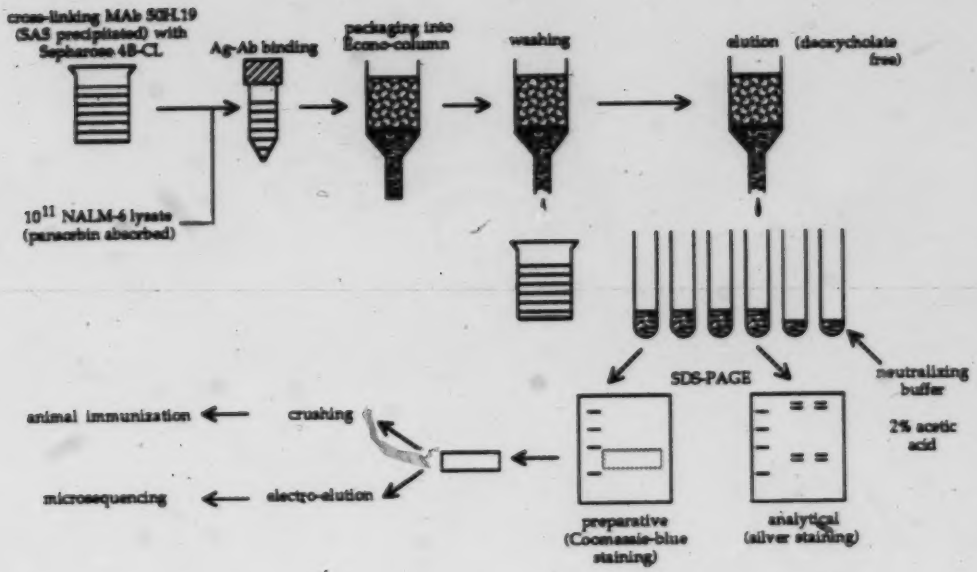


FIGURE 3. Purification of CD9 antigen by mAb affinity chromatography. NALM-6 cells were lysed, clarified, and the non-specific binding components were removed by absorption with Pansorbin. The lysate was then mixed with the mAb 50H.19 cross-linked to the protein A-Sepharose CL-4B in 50 ml tubes, and the tubes rotated at 4°C overnight. The beads were then transferred into an 8 ml econo-column. The column was washed extensively with buffer, and the antigen eluted in 1 ml fractions with 0.05 M diethylamine pH 11.5 (without deoxycholate) into Eppendorf tubes containing 1/10 volume of 2% acetic acid. Sample from each fraction was analyzed by SDS-PAGE followed by silver staining. The fractions containing the CD9 antigen were pooled, concentrated, and the proteins separated by preparative SDS-PAGE. The gel band containing the antigen was excised, and used either for animal immunization, or for further purification by electroelution.

Figure 4. Analysis of CD9 antigen from the platelets of 10 individuals by Western blotting. Lysates were prepared from human platelets obtained from healthy volunteers, denatured by boiling in the presence of SDS, and separated by SDS-PAGE on a 12.5% acrylamide gel. The proteins were blotted onto a nitrocellulose filter. CD9 was detected with 1/200 dilution of the mAb 50H.19 followed by incubation with horseradish peroxidase conjugated goat anti-mouse second antibody. All platelets expressed 22, 24, 27, and 28 kDa components. Interestingly, there was donor variation in the relative intensity of the components, and platelets from two of the ten donors expressed an additional 20.5 kDa protein which may be the nonglycosylated precursor of CD9 (Lane a and e). A protein with a molecular weight of 18.5 kDa was seen in every lane, which probably resulted from proteolysis.

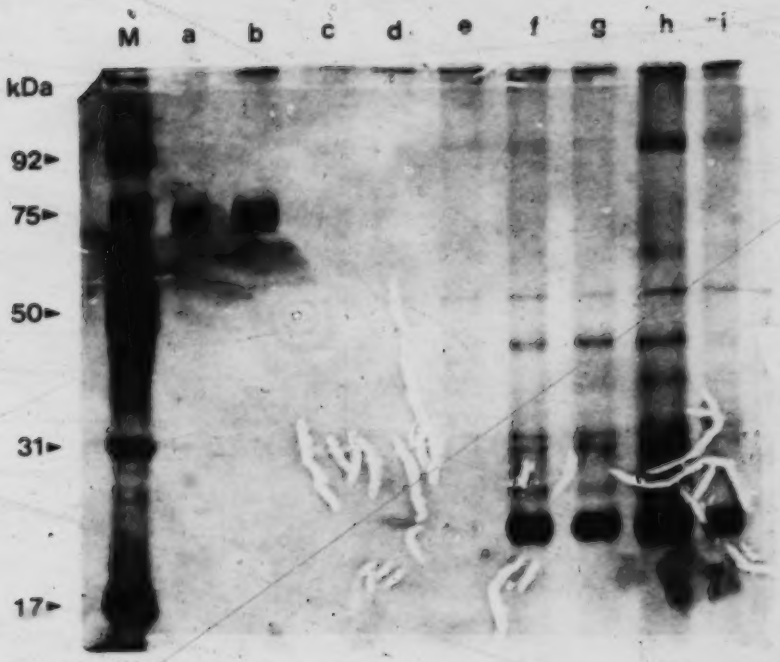


FIGURE 5. Analysis of the CD9 antigen purified from human platelets by SDS-PAGE. Platelet lysate was purified by the mAb 50H.19 affinity silver staining as in Figure 2, except 10 µl from each fraction of unconcentrated column eluate was used. Lane a and b were loaded with BSA protein standard with 0.5 µg and 1.0 µg in each respectively. Lane c to Lane h were loaded with the eluate from fraction 2 to 9. CD9 antigen was present in Lane f to Lane i. In relation to the BSA protein standard, there appeared to be about 2 µg, 2 µg, 4 µg, and 1 µg of CD9 antigen respectively in each of these four lanes. Although CD9 was the major protein in the column eluate (including the major gp24 component and the minor components of gp24 and gp27) minor proteins of 39 kDa, 45 kDa, 54 kDa, 60 kDa, and 92 kDa respectively were also detected.

blood was collected from each of 10 healthy volunteer donors, and a platelet lysate was prepared as described in previous chapter. 3 x 10^8 platelets were lysed in 0.1 ml of 20 mM Tris-HCl pH 8.0, 1 mM Na_2HPO_4, 0.137 M NaCl and 0.5% NP-40, and after clarification and absorption with Pansorbin, 10 µl was taken from each sample, and analyzed for the amount of CD9 antigen by Western-blotting. The nitrocellulose filter was incubated with 1/400 dilution of the mAb 50H.19, followed by incubation with 1/3000 dilution of the GAM-HRP second antibody. Figure 4 is the result of the Western blotting. All platelets from the 10 donors strongly expressed the CD9 antigen. Interestingly, two of the donors possessed, in addition to the mature CD9 components, a smaller component of 20.5 kDa. The significance of this band is not known.

Having confirmed the abundance of CD9 antigen in human platelets, large scale purification was carried out with out-dated platelets from the Red Cross Center. 50 to 60 bags (50 ml of concentrated platelets per bag, isolated from 500 ml of peripheral blood) of the out-dated platelets were used each time to bind the 7 ml of the mAb 50H.19-protein A-Sepharose CL-4B, and the elution performed according to the modified procedure, using the deoxycholate-free solution. In addition, all the laboratory ware used in the processes was pre-incubated with the straight platelet lysates. The antigen preparation, antigen coupling, column elution, and eluate analysis as well as column reconstitution were performed according to the NALM-6 purification described above, with the exception that the eluate was analyzed without concentration. Figure 5 illustrates the purity and the concentration of the CD9 protein isolated from platelets. CD9 was detected in four fractions. Based on the BSA protein standard, the concentration of CD9 protein in each fraction was estimated to be 200 µg/ml, 200 µg/ml, 400 µg/ml, and 100 µg/ml respectively. Compared to the CD9 antigen purified by affinity chromatography from NALM-6 cells, the antigen isolated from platelets seemed less 'clean', since several other minor protein bands could be seen (p92, p60, p54, and p39). About 1 to 1.5 mg

of column purified CD9 protein could be recovered from each 50 to 60 bags of out-dated human platelets.

7. *Immunization of Rabbits with CD9 Antigen Isolated from Platelets*

Initial rabbit immunization was carried out using the crushed gel strips containing the 22 kDa component of CD9 antigen (10 μg per injection) isolated from the NALM-6 cells. However, no high titre antibody was detected even 126 days after the first injection. Column eluate containing CD9 antigen isolated from the out-dated platelets was then used to immunize a second rabbit. The antigen was denatured by boiling in the presence of SDS and 2ME, mixed with Freund's adjuvant, and injected at multiple subcutaneous sites. 100 μg of the CD9 protein was used for each of the first four injections. 5 ml of the peripheral blood was obtained before each injection, and was tested for reactivity to CD9 antigen by ELISA. Low titre antisera (1/32 dilution) was detected after the fourth injection (63 days after the first injection). Therefore, the amount of antigen used for the subsequent injection was increased to 250 μg. High titre antibody (1/2048 dilution) to CD9 antigen was detected on the sixth injection (105 days after the first injection). 30 ml of blood was obtained on the following day. The immunization schedule is summarized in Table 4. The antisera was prepared and stored as described before.

8. *Evaluation of the Rabbit Polyclonal Antibody*

To determine the specificity of the polyclonal antibody, cell lysates were prepared from the surface CD9 positive cell lines NALM-6, SKOSC and HeLa cells, and the reactivity of the polyclonal antibody against CD9 antigen tested by Western blotting. The nitrocellulose filter was incubated with a 1/400 dilution of the antisera, and then 1/3000 dilution of the GAR-HRP second antibody. Figure 6 shows the result of the Western blotting. That the polyclonal antibody was able to recognize the CD9 antigen was demonstrated by the ability of the former to bind to denatured CD9 protein purified from

TABLE 4

SCHEDULES OF RABBIT IMMUNIZATION

First Injection	Day 0
Amount of Ag	100 μg
Adjuvant	Complete
Second Injection	Day 21
Amount of Ag	100 μg
Adjuvant	Incomplete
Third Injection	Day 42
Amount of Ag	100 μg
Adjuvant	Incomplete
Fourth Injection	Day 63
Amount of Ag	100 μg
Adjuvant	Incomplete
Fifth Injection	Day 84 *
Amount of Ag	250 μg
Adjuvant	Incomplete
Sixth Injection	Day 105
Amount of Ag	250 μg
Adjuvant	Incomplete

*The rabbit was initially injected subcutaneously with column eluate containing 100 μg of protein per injection. On day 84 at the fifth injection, the antigen dosage was increased to 250 μg per injection. The immunization resulted in high titre antibody by the sixth injection (day 105).

The rabbit was boosted with a further injection of antigen 21 days before every subsequent bleeding.

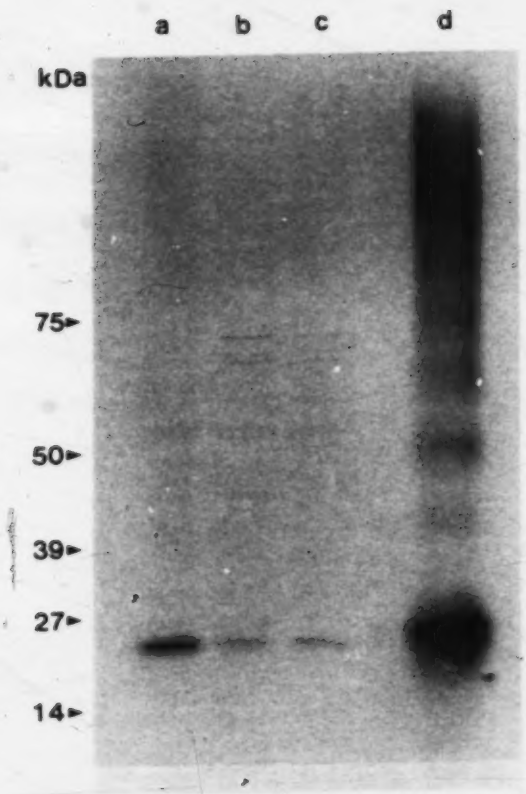


Figure 6. Analysis of the CD9 polyclonal antibody by Western blotting. Cell lysates were prepared from NALM-6, Skosc, and Hela cells. CD9 protein (gp22) purified from human platelets by mAb 50H.19 affinity chromatography and further purified by electroeluting the 22 kDa component was included as positive control protein. The proteins were separated by SDS-PAGE using 5% to 20% acrylamide gradient gel and blotted onto a nitrocellulose filter. The filter was incubated with 1/400 dilution of the polyclonal antibody overnight at 4°C, washed with PBS, then incubated with 1/3000 dilution of horseradish peroxidase conjugated goat anti-rabbit second antibody at room temperature for 2 hours. The filter was finally developed in 3-amino-9-ethylcarbazole color substrate. Lane a, b, and c were loaded with the lysate from NALM-6, Skosc, and Hela cells respectively, while Lane d was loaded with the CD9 protein (gp22). The polyclonal antibody reacted strongly with the mAb purified CD9 (gp22) (Lane d). The minor bands in the same lane loaded with the electroeluted CD9 (gp22) may due to the formation of CD9 aggregates. Furthermore, the polyclonal antibody also recognized a major protein of 22 kDa in lysates from NALM-6 (Lane a), Skosc (Lane b), and Hela (Lane c) cells, and a minor protein of 24 kDa in lysate from NALM-6 cells (Lane a). The polyclonal antibody did not react with other proteins from these cells.

Figure 7. Analysis of the protein immunoprecipitated with polyclonal antibody by V8 peptide mapping. NALM-6 cells were surface labeled with ^{125}-I. Proteins immunoprecipitated with polyclonal antibody from the labeled cell lysates were separated by SDS-PAGE and the band containing the 22 kDa protein was excised. The protein was subjected to cleavage by V8 protease and the proteolytic products were then separated again by SDS-PAGE. Following fluorography, the V8 cleavage pattern of the protein immunoprecipitated with polyclonal antibody was then compared to the similarly cleaved CD9 protein (gp22) immunoprecipitated with CD9 mAb 50H.19. Lane a was loaded with the cleaved CD9 immunoprecipitated with mAb 50H.19, while Lane b was loaded with the cleaved 22 kDa protein immunoprecipitated with the polyclonal antibody. Both proteins possessed a same V8 cleavage pattern.

human platelets by CD9 mAb 50H.19 affinity chromatography and further purified by electroelution. The polyclonal antibody also reacted with a major protein of 22 kDa in lysates from NALM-6, SKOSC and HeLa cells, and a minor protein of 24 kDa in lysate from NALM-6. The ratio of the major and the minor proteins recognized by the polyclonal antibody was very similar to the CD9 protein recognized by the CD9 mAb 50H.19, suggesting that they were in fact CD9 proteins. Furthermore, the antibody displayed a high specificity against CD9 antigen, with little if any cross reactivity against other proteins in these cells. To further investigate whether the protein recognized by the polyclonal antibody in Western blotting was CD9 antigen, the polyclonal antibody was used to immunoprecipitate protein from NALM 6 cells which was further analyzed by limited proteolytic cleavage by V8 protease followed by SDS-PAGE (performed by Dr. Jutta Seehafer). Following fluorography, the V8 proteolytic pattern of the protein immunoprecipitated by the polyclonal antibody was then compared to the CD9 protein immunoprecipitated by mAb 50H.19 and similarly cleaved with V8 protease. As presented in Figure 7, the protein immunoprecipitated by the rabbit polyclonal antibody gave rise to a V8 cleavage pattern identical to the CD9 antigen immunoprecipitated by mAb 50H.19. Finally, the ability of the polyclonal antibody to recognize native CD9 protein was tested by incubating it with surface CD9 negative and positive cells, and examination by indirect immunoflorescence microscopy, and FACS analysis. While 0% of the surface CD9 negative Raji cells were positive, 40% of the surface CD9 positive NALM-6 cells and 16% of the activated T cells displayed specific antibody bindings.

C. DISCUSSION

An important step in monoclonal affinity chromatography is the cross-linking of the antibody to the solid matrices. The quality of the cross-linking between monoclonal antibody and protein A-Sepharose CL-4B determines the 'longevity' of the column, and

depends not only on the nature of the cross-linking reaction but also on the amount of antibody used per mg of the Sepharose beads. Over-saturation of the protein A binding sites would result in wastage of the antibody, and possible inhibition of the cross-linking due to excess proteins. Under-saturation of the protein A binding sites on the other hand would waste the Sepharose, and might promote non-specific protein binding to the unoccupied protein A. The protein A content of the swollen gel is 2 mg/ml and the binding capacity for immunoglobulin is approximately 25 mg/ml gel. Although optimal antigen binding was found to occur at 50% saturation (about 12 mg antibody per ml of packed beads) in the original report by Schneider et al. (1982), the antibody used in their study was affinity-purified (on a mouse Ig-Sepharose 4B affinity column), whereas mAb 50H.19 was only partially purified (by ammonium sulfate precipitation). Before large scale cross-linking was carried out, it was necessary to determine the optimal amount of the monoclonal antibody to be used. However, since the supply of the mAb 50H.19 was very limited (in fact, the amount of the antibody was only enough to make one column at that time), it was impossible to carry out a systematic study to determine this optimal ratio. The partially purified antibody was therefore used as the purified antibody, and if the partially purified mAb 50H.19 was assumed to contain about 20% mouse immunoglobulin, the amount of the antibody used per ml of the packed beads would result in 28% saturation, an acceptable saturation without sacrificing specificity. Although this ratio may result in some wastage of the Sepharose beads, it did ensure maximal utilization of the precious monoclonal antibody evidenced by the lack of immunoglobulin bands in the buffer after the antibody coupling.

The amount of cell lysate used per ml of packed Sepharose beads should also be titred, in order to avoid lysate wastage in the case of antigen excess, or non-specific binding of the proteins in the case of Sepharose excess. However, at the time CD9 antigen was isolated from the NALM-6 lysate, the technique of Western blotting was not

yet adopted in the laboratory. Therefore, residual CD9 antigen could not be estimated following antibody-protein A-Sepharose adsorption. Although small scale coupling using the ^{125}I-labeled antigen could be used to find out the proper lysate/beads ratio, the information may not be applicable to large scale purification. In fact, since the supply of the monoclonal antibody was the limiting factor in the large scale purification, it was better to oversaturate the antibody binding sites. Therefore, for each large scale purification, excess lysate was used for antigen-antibody-protein A-Sepharose coupling.

The stickiness of the CD9 antigen may be explained by its fatty acid component. In fact, considering the role played by CD9 in cell-cell or cell-environment interaction, this would be an expected property of the molecule, since CD9 is known to possess multiple acylation sites. The fatty acid component would render the molecule more hydrophobic, enhancing its interaction with other hydrophobic molecules, and even among itself. Indeed, when the pure CD9 antigen (22 kDa), isolated by SDS-PAGE followed by electroelution, was denatured (boiling in the presence of 2ME) and analyzed again by SDS-PAGE, a new 47 kDa band was observed (see later chapter). This is likely a CD9 dimer that formed after the electroelution, which could no longer be dissociated.

The hydrophobic nature of the CD9 antigen might also explain the difficulty experienced in separating the antigen from the detergent deoxycholate. Detergent is known to form micelles with hydrophobic molecules, and these micelles cannot be disrupted by simple physical measures such as dialysis, and ultra-filtration. Deoxycholate itself is known to become viscous and gel when the pH of the solution falls below 8 or if the ionic strength of the solution is too high. Furthermore, being ionic, it affects the mobility of the proteins during electrophoresis (Dalchau and Fabre, 1982). Since it was the pH and salt concentration changes that dissociated the antigen from the antibody, the presence or absence of the detergent would not be expected to have much effect on the elution efficiency of the high pH (pH 11.5) buffer. As

evidenced from the pilot affinity chromatography with the ^{125}I-labeled NALM -6 cells, removal of deoxycholate from the elution solution only resulted in a slight decrease in antigen recovery from the column. On the other hand, the absence of the detergent made concentration of antigen feasible, which was a necessary step in order to visualize the antigen by SDS-PAGE followed by silver staining (although no concentrating was necessary when platelets were used). The replacement of bicarbonate with acetic acid as the column eluate neutralization solution also avoided salt precipitation during concentration.

Although coating with BSA greatly reduced the non-specific binding of the CD9 antigen, the trace BSA present in the column eluate posed a problem, since CD9 would stick to BSA and make it very difficult to separate the two proteins, even by reverse phase HPLC. Coating with the cell lysate proved to be a superior method, since there was no exogenous protein. In addition, the hydrophobic proteins in the lysate other than the CD9 protein would also act as blocking agents, making the lysate a better choice. The fact that 0.7 mg of CD9 antigen was isolated from 1 x 10^{11} NALM-6 cells using the coated tubes whereas no antigen was detected in the eluate from the same number of cells when uncoated tubes were used clearly indicates the importance of the coating.

High titre polyclonal antibody against CD9 antigen was successfully raised using the mAb 50H.19 affinity column eluate from platelet lysates. Despite some other coeluting components in the column eluate, CD9 antigen was the predominant one. Since more CD9 protein would be lost with further purification procedures, the column eluate was therefore used directly as the immunogen. However, because of the coeluting components, it was very important to determine if the polyclonal antibody recognized the CD9 antigen. The ability of the polyclonal antibody to recognize CD9 protein was demonstrated by the Western blotting, in which it bound to the CD9 antigen purified from human platelets by mAb 50.19 affinity chromatography and further purified by

electroeluting the gp22 CD9 protein. The polyclonal antibody also recognized a major protein of 22 kDa from CD9 producing NALM-6, SKOSC, and HeLa cells, and a minor protein of 24 kDa. The ratio of the two proteins from NALM-6 lysate recognized by the polyclonal antibody is very similar to the CD9 protein recognized by the CD9 mAb 50H.19. That the 22 kDa protein indeed represented the CD9 antigen was confirmed by the peptide mapping experiment, which demonstrated that this protein possessed a V8 proteolytic cleavage pattern identical to that obtained from the CD9 antigen immunoprecipitated by CD9 mAb 50.19. In addition, the polyclonal antibody produced a very "clean" Western blot in which only CD9 protein bands were visualized in cell lysates from NALM-6, SKOSC, and HeLa cells. This strongly indicated that the polyclonal antibody was not only able to recognize the CD9 antigen, it was also able to do so specifically. This was possible since the coprecipitates only comprised a very minor proportion of the total proteins. The antibody developed against the coprecipitates would therefore be expected to be of very low titre. The specificity of the polyclonal antibody was further supported by indirect fluorescence microscopy and by the FACS analysis experiments, which showed that the antibody only bound to surface CD9 positive cells, not to the surface CD9 negative cells. These experiments, together with a chemical cross-linking experiment carried out in our laboratory in which the polyclonal antibody precipitated a 22 kDa protein in combination with the specifically CD9-associated GP IIb/IIIa complex, proved beyond any doubt that the rabbit polyclonal antibody specifically recognized CD9 protein.

Finally, the fact that only 40% NALM-6 cells and 16% activated T-cells were stained with the polyclonal antibody may be explained by the ability of the antibody to preferentially recognize the denatured CD9 protein. Since the rabbit was immunized with the denatured and reduced CD9 antigen, the polyclonal antibody would be expected to react only to the linear epitopes of the protein. When the surface CD9 bearing cells were

stained with the polyclonal antibody, a lot of these linear epitopes may be inaccessible due to protein folding. The polyclonal antibody would therefore have a low affinity to the native CD9 protein, which may result in only a proportion of the cells being stained. The difference in the percentage of cells which scored positive for polyclonal antibody staining between NALM-6 and the activated T-cells may be explained by the fact that activated T-cells express lower level of surface CD9 antigen, and that they also contain resting T-cells and possibly other surface CD9 negative cells of different lineages.

CHAPTER IV

MOLECULAR CLONING AND SEQUENCING OF CD9 cDNA

A. INTRODUCTION

The ability to clone DNA from any organism probably represents the major breakthrough of the decade in molecular biology. Pioneered by Paul Berg, Herbert Boyer, and Stanley Cohen in early 1970s (Jackson et al., 1972; Cohen et al., 1973; Cohen, 1988), the recombinant DNA technology has revolutionized biological research by providing novel means of analyzing and altering genes and proteins. Single discrete segments of DNA from a population of genes can be purified to homogeneity, and amplified, producing enough pure material for chemical, genetic, and biological analysis (DNA cloning). The nucleotide sequence of the cloned DNA can rapidly be determined, leading to the prediction of the primary (amino acid sequence) and secondary structure of the encoded protein (Sanger et al., 1977; Gilbert, 1981). Sequence comparison with other known proteins and/or genes would indicate the possible function and/or the evolution of the encoded protein. Furthermore, radiolabeling of the purified DNA would allow copies of related DNA sequences or mRNA to be isolated, and analyzed. In addition, cloning of the genomic DNA with the purified cDNA as a probe would provide information on the structure and expression control of this particular gene (Goeddel et al., 1981). Finally, engineering of the cloned DNA in bacteria and yeast may allow expression of the encoded protein, providing an inexpensive and abundant source of otherwise unattainable proteins of biological or medical importance (Goeddel et al., 1979). Expression of the altered DNA in cells or in whole animals also enables the observation of the changes produced by this mutant protein, resulting in a better understanding of its physiological function (Anderson and Diacumakos, 1981).

One of the major problems encountered in DNA cloning is the identification of clones of interest. Many ingenious methods have been developed to screen for specific DNA clones in cDNA libraries. Broadly, these methods rely either on a DNA or RNA probe to select the corresponding cDNA through *in situ* hybridization, or on the detection of the gene product by either functional assay, or antibody recognition. In the first approach, probes are commonly made from an mRNA preparation that contains high levels of the sequence of interest through hybrid-selected translation (Harpold et al., 1978; Ricciardi et al., 1979) or hybrid-arrested translation (Paterson et al., 1977), this high level of specific mRNA either existed physiologically, or is enriched by physical fractionation such as sedimentation and gel electrophoresis, or by immunoprecipitation of polysomes. Alternatively, differential hybridization (or 'plus and minus screening', St. John and Davis, 1979) or subtractive hybridization (Taniguchi et al., 1979) can be used to generate cDNA probes if two mRNA populations are available that differ principally in their content of the mRNA of interest. These probes are difficult to generate, however, when the mRNA is in short supply (e.g., when cloning a rare mRNA), and when the efficiency of *in vitro* translation is low (e.g., for large mRNA molecules), when the two mRNA populations differ in more than a few mRNAs (as is always the case). In addition, if the partial amino acid sequence of the protein is known, oligonucleotide probes can be chemically synthesized and used for library screening (Suggs et al., 1981). The second approach involves cloning the cDNA into an expression vector that promotes expression of the cDNA in E. coli. The resultant 'expression library' then is screened for appropriate translation products by using either a functional (Struhl et al., 1976) or an immunological assay (Broom and Gilbert, 1978). The second approach bypasses the need for generation of DNA probes and provides promising protocols for the cloning of cDNAs from low abundance mRNAs. An

efficient expression vector is clearly also essential for identifying the specific cDNA clones.

The expression in E. coli is normally accomplished by inserting the cDNAs into plasmid or bacteriophage vectors within the genetic regions that can be highly expressed once the vectors are introduced into a host bacterium. This is effected by placing the eukaryotic sequences downstream from a strong bacterial, or phage promoter. Since the high level production of foreign proteins may block the growth of the host, the promoters chosen for expression vector can usually be regulated. In addition, to ensure efficient translation, a ribosomal binding site is often included. A more convenient approach to achieve efficient and regulated expression as well as efficient translation is to insert the cDNA inside a regulated gene of the plasmid or phage vector. The cDNA encoded protein is then expressed as a part of the regulated plasmid or phage protein (fusion protein). The expression of the cDNA as a fusion protein not only secures the production of the foreign protein, it also prevents the degradation of the expressed protein, since the bacterial host treats the fusion protein as one of its own proteins (Itakura et al., 1977).

Of the various vectors and procedures developed to construct expression libraries and to detect the expressed proteins in bacterial colonies and bacteriophage plaques (Skalka and Shapiro, 1976; Sanzey et al., 1976; Erlich et al., 1978; Broom and Gilbert, 1978; Villa-Komaroff et al., 1978; Hitzeman et al., 1980; Kemp and Cowman, 1981; Grey et al., 1982; Hefman et al., 1983; Ruther and Muller-Hill, 1983; Weinstock et al., 1983; Stanley and Luzio et al., 1984), the expression system developed by Young and Davis (1983 a and b) has emerged over the past 5 years as the method of choice for cDNA cloning with antibody. In this system, a bacteriophage λgt11 was engineered to permit the construction and maintenance of large cDNA libraries. Propagation of the recombinant phage in the host cell as a single-copy genomic insert enhanced its stability

and facilitated the reexpression of the foreign protein. Insertion of the cDNA into the lac-Z gene enabled the recombinant phage to be distinguished from the wild type phage by a color reaction, since while the wild type phage with the intact lac-Z gene would produce β-galactosidase which generated blue plaques in the presence of color substrate, the cDNA carried by the recombinant phage would inactivate the lac-Z gene resulting in white plaques. High levels of transcription could be achieved by fusing the cDNA encoded protein with β-galactosidase and inducing with isopropyl-β-D-thiogalactopyranosine (IPTG). The expression in the form of fusion protein and in a *lon* protease deficient *E. coli* Y1090 (Δ*lon*) greatly reduced the degradation of the foreign protein. In addition, restriction of foreign DNA prior to host modification was prevented by first amplifying the library in *E. coli* Y1088 (*hsd*R$^-$*hsd*M$^+$), and the production of large amounts of fusion protein was made possible by lysogenizing in *E. coli* Y1089 deficient in amber suppressor *sup*F (necessary for complementing the phage's amber mutation in order to produce lytic plaques). Finally, the temperature-sensitive repressor (*c*I857) also enabled the lysis to occur at 42°C (to visualize the individual phage plaque), while suppressing the phage lytic growth at 37°C (when protein expression was induced for antibody screening).

The λgt11 expression system was therefore chosen to construct the NALM-6 cDNA library. Both the mAb 50H.19, and the polyclonal anti-CD9 antibody developed later, were used to screen the NALM-6 cDNA library and the HepG2 cDNA library. The result and discussion of the molecular cloning and sequencing of CD9 cDNAs are presented in this chapter, while further analysis and characterization of the CD9 cDNA are discussed in the following chapters.

B. RESULTS

1. *NALM-6 λgt11 Library Construction*

The construction of the NALM-6 λgt11 cDNA library is summarized in Figure 8. The yield of the first strand cDNA synthesis from 10 μg of poly A^+ mRNA was calculated by counting the radioactivity of the first peak collected from the column, which varied from 3 μg to 6 μg. Following poly-dG tailing, the second strand cDNA was synthesized using the oligo-dC primer. After methylating the internal Eco RI sites, followed by Eco RI linker ligation and Eco RI cleavage, the double stranded cDNAs were size selected by 0.8% agarose gel electrophoresis. Gels containing the cDNAs longer than 800 base pairs were excised (Figure 9), the cDNAs were recovered from the gels by the CETAB procedure (Langridge et al., 1980), and the radioactivity remaining was again counted. About 600 ng of ds cDNA (greater than 800 bp in length) was usually recovered for use in the subsequent ligation and *in vitro* packaging. From the initial 10 μg of poly A^+ mRNA, a NALM-6 λgt11 cDNA library containing 4×10^5 recombinant phage were obtained. Plating a fraction of the amplified (in E. coli Y1088) library on a X-gal LB plate (pH 7.5) in the presence of IPTG revealed that 80% of the phage were recombinants, as indicated by the number of white plaques among the total plaques. Phage DNAs were isolated from 10 randomly picked white plaques, and analyzed by digestion with Eco RI followed by 0.8% agarose gel electrophoresis. 9 out of the 10 phage contained cDNA inserts, which varied between 800 to 1000 bp in length.

2. *Comparison of the Various Antigen Detection Methods*

The recombinant phage λSCS8 was plated out on small LB plates (pH 7.5), and the fusion protein was detected by incubation with the 1/200 dilution of the rabbit polyclonal antibody. The nitrocellulose filters were blocked either with 20% fetal calf serum or with 3% gelatin, and the specifically bound first antibodies were visualized

either by ^{125}I-protein A (1 x 10^6 c.p.m./ 82 mm filter) or by horseradish peroxidase conjugated goat anti-rabbit antibody (HRP-GAR) (1/3000 dilution) in the presence of color substrate, as summarized in Figure 10. The results from these experiments are presented in Table 5. As indicated from the study, blocking with gelatin resulted in less non-specific binding to the filters of the first antibody, and/or the protein A or second antibody than did blocking with FCS. Furthermore, replacement of the Bio-Rad color reagent with the 3-amino-9-ethylcarbazole (prepared as described in Materials and Methods) enhanced the intensity of color reaction in both situations. Finally, the positive signals detected by ^{125}I-protein A were much stronger than those detected by the HRP-GAR second antibody, although, the negative signals from the background (or antigen negative) phage plaques were also significantly higher. Since the gelatin blocking, combined with visualization by the HRP-GAR antibody in the presence of the 3-amino-9 ethylcarbazole, gave the best ratio of strong positive to weak negative signals, this method was chosen to carry out the library screening.

3. *Minimizing Polyclonal Antibod : Cross Reaction to E. Coli Protein*

Having decided the proper antibody screening method, an experiment was performed to determine the optimal dilution of CD9 polyclonal antibody. 1000 p.f.u. phage from the NALM-6 λgt11 library were plated out on each small LB plate (pH 7.5). After incubation, induction, and transfer, the nitrocellulose filters were blocked with gelatin, and incubated with various dilutions of CD9 polyclonal antibody. All the filters were then incubated with 1/3000 dilution of HRP-GAR second antibody, and followed by 3-amino-9-ethylcarbazole color reagent treatment. Table 6 summarizes the effect of first antibody dilution on the background signals. As concluded from the study, 1/400 dilution of the first antibody was chosen to determine the effect E. coli protein had on the background signals.

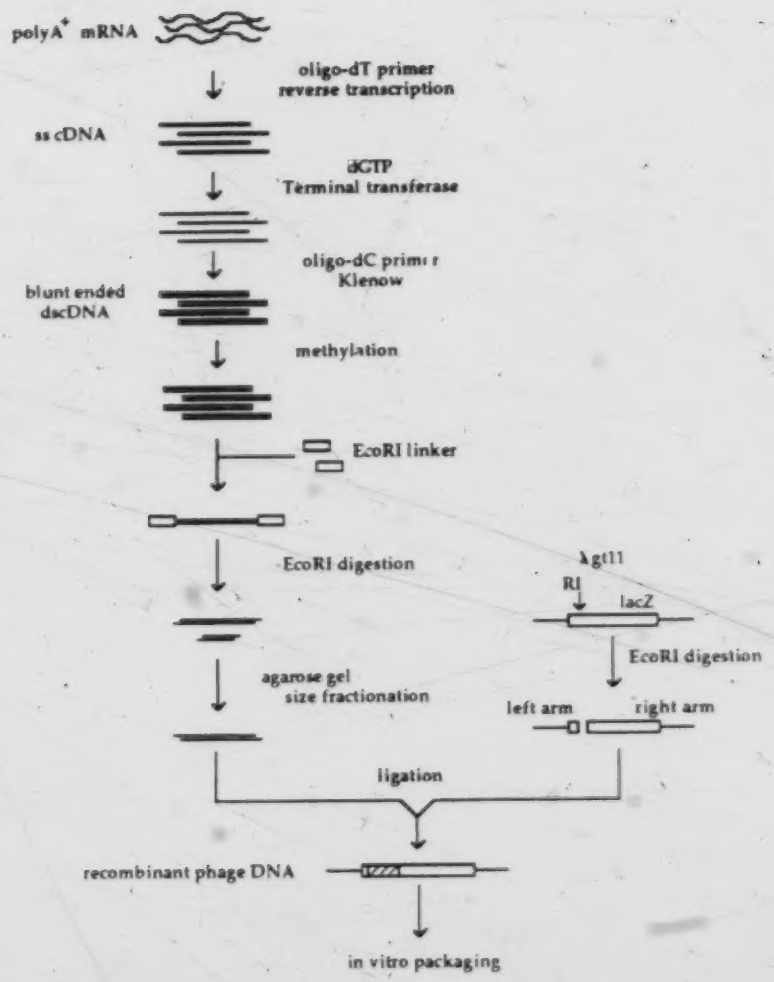


FIGURE 8. Construction of the NALM-6 λgt11 cDNA library. 10 μg of poly A^+ mRNA was reverse transcribed with AMV reverse transcriptase into single stranded cDNA (ss cDNA) using oligo-dT as primer. poly dG tail was added to the 3' end of the first cDNA strand by terminal transferase, and the second cDNA strand was synthesized by DNA polymerase (Klenow fragment) using oligo-dG as primer. The internal Eco RI sites on the cDNA were protected with Eco RI methylase treatment, after which Eco RI linker was added to the both ends of the double stranded cDNA (ds cDNA) by T4 DNA ligase. Eco RI sticky ends were created by digestion of the cDNA with Eco RI, and the cDNAs were size-selected by agarose gel electrophoresis. The cDNA with desired length were recovered by the 'CETAB' method, and ligated to the Eco RI cleaved λgt11 DNA arms. The recombinant phage DNAs were in vitro packaged using the purchased phage packaging extracts.

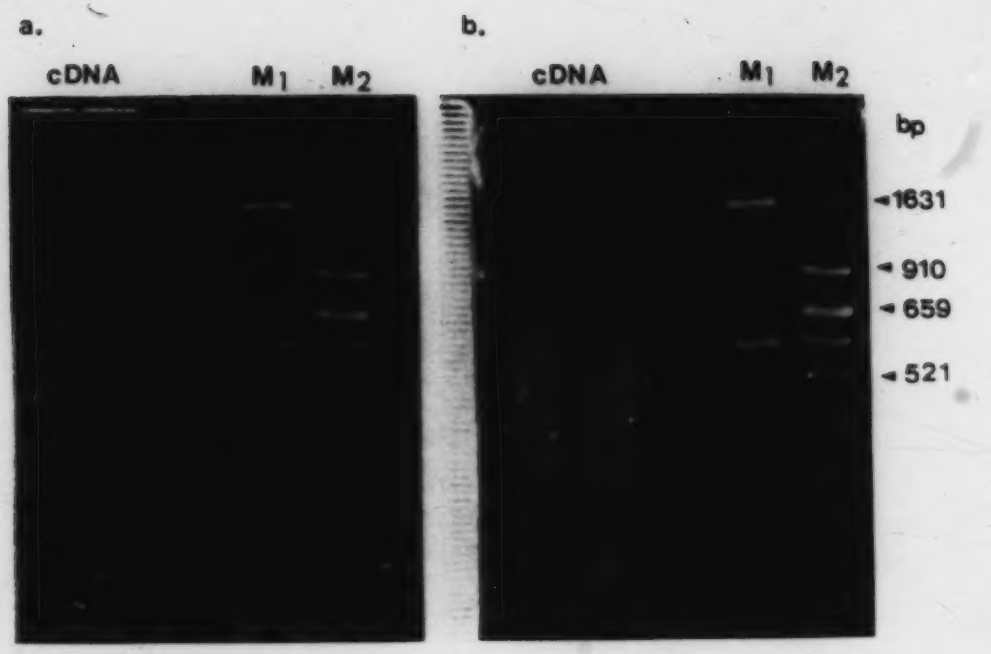


Figure 9. Size fractionation of the cDNAs by agarose gel electrophoresis during library construction. The Eco RI digested cDNAs were mixed with 10 µl of 10 x DNA loading dye, and separated by electrophoresis on a 1.4% low melting point agarose gel. The DNA was visualized by staining with 0.5 µg/ml of ethidium bromide in water for 30 minutes followed by destaining in water for 15 minutes. The gel containing the cDNAs longer than 800 bp was excised, and the DNA recovered by the 'CETAB' method. Figure 7 a and b are the pictures taken before and after the gel was excised. Lane M_1 was loaded with the DNA size markers (pBR322 DNA digested with HinF I) and Lane M_2 was the pBR322 DNA digested with Alu I.

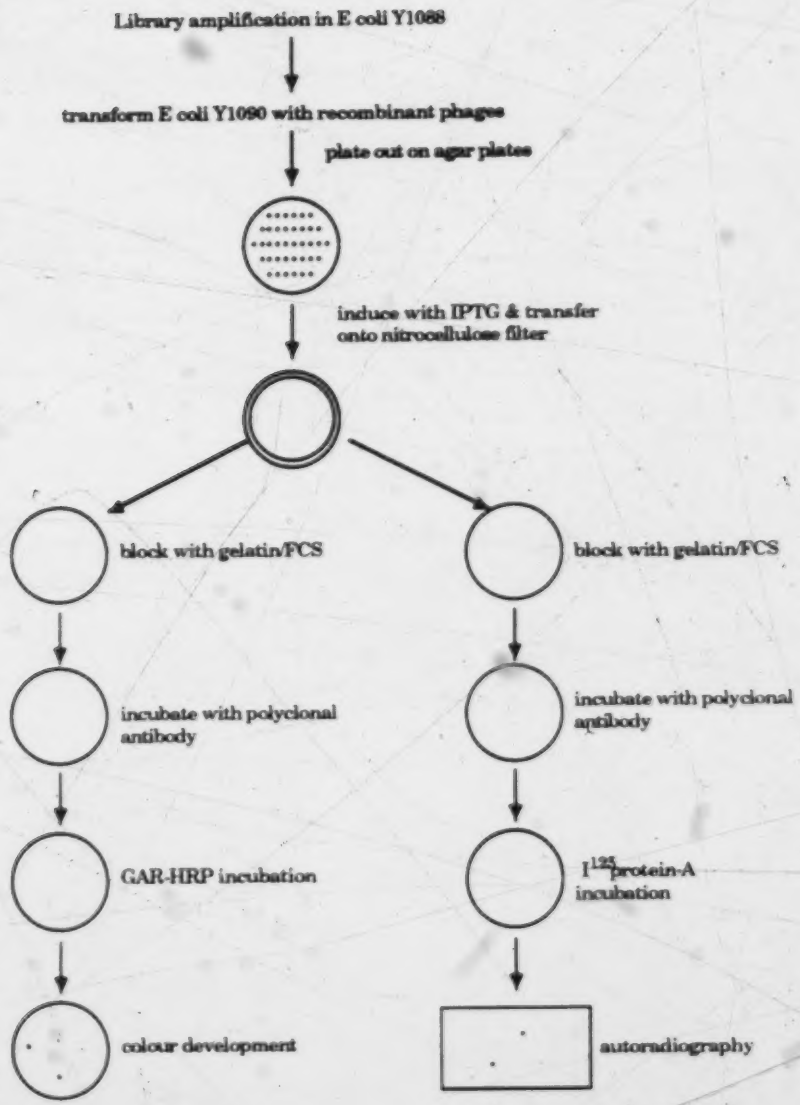

Library amplification in E coli Y1088
transform E coli Y1090 with recombinant phages
plate out on agar plates
induce with IPTG & transfer onto nitrocellulose filter
block with gelatin/FCS
block with gelatin/FCS
incubate with polyclonal antibody
incubate with polyclonal antibody
GAR-HRP incubation
I125 protein-A incubation
colour development
autoradiography

Figure 10. Summary of the different library screening methods using antibody probe. Recombinant λgt11 phage were plated out on LB plates (pH 7.5). After 4 to 5 hours' incubation at 42°C, nitrocellulose filters saturated with IPTG were used to induce, and blot the fusion proteins, and to blot the fusion proteins. The filters were washed with TBS, blocked with 20% fetal calf serum, or with 3% gelatin for 30 minutes, incubated with 1/200 dilution of the polyclonal antibody overnight at 4°C, washed with TBS and T-TBS, and the antibodies visualized with ^{125}I-labelled protein A or with horseradish peroxidase conjugated goat anti-rabbit second antibody.

TABLE 5

COMPARISON OF DIFFERENT ANTIBODY SCREENING METHODS

Blocking Agent	First Antibody Detecting Agent	Color Reagent	Signal* positive	negative	background
20% FCS	^{125}I-Protein A		++++	+++	++
3% Gelatin	^{125}I-Protein A		+++	++	+
20% FCS	HRP-GAR Ab +	Bio-Rad Substrate		+++	++
3% Gelatin	HRP-GAR Ab +/-	Bio-Rad Substrate		++	+/-
20% FCS	HRP-GAR Ab	AEC	++++	+++	++
3% Gelatin	HRP-GAR Ab	AEC	+++	+	-

Nitrocellulose filter imprints from the LB plates containing a mixture of λSCS8 and wild-type λgt11 phage plaques were blocked with either 20% fetal calf serum (FCS) or 3% gelatin, incubated with the same antibody (1/200 dilution), and then with either ^{125}I-protein A or horseradish peroxidase conjugated goat anti-rabbit (HRP-GAR) antibody. The positive signals were detected by autoradiography, or the Bio-Rad color substrate, or 3-amino-9-ethylcarbazole (AEC).

*The strength of the signal is expressed in relative terms only. The signal strength from ^{125}I-protein A cannot be compared to that from HRP-GAR Ab, since the the former is detected by autoradiography, while the latter is detected by color reaction.

TABLE 6

EFFECTS OF ANTIBODY AND E. COLI LYSATE CONCENTRATION ON SIGNAL TO NOISE RATIO*

1. Effect of Varying Antibody Concentration§

Antibody Dilution	1/100	1/200	1/400	1/800	1/1600
Background Signal	++	+	+	+/-	+/-

2 Effect of Varying E. coli Lysate Concentration†

Antibody Dilution	1/400	1/400	1/400	1/400	1/400
E. Coli Concentration	1/50	1/100	1/200	1/400	1/800
Background Signal	-	+/-	+/-	+	+

§Nitrocellulose filter imprints from the LB plates containing NALM-6 λgt11 phage plaques were blocked with 3% gelatin, incubated with the variably diluted CD9 polyclonal antibody, and then with horseradish peroxidase conjugated goat anti rabbit (HRP-GAR) antibody. The signals were visualized with the 3-amino-9-ethylcarbazole color substrate.

†The experiment was repeated, except the filters were incubated with 1/400 dilution of CD9 polyclonal antibody preadsorbed with varying concentrations of E. coli lysate.

*The signal strength is expressed in relative terms only.

To test the optimal amount of E. coli lysate needed to reduce the background signals due to cross reactive rabbit antibody against E. coli protein, the diluted first antibody (1/400 dilution) was first adsorbed with various concentrations of E. coli lysate, and the pre-treated antibody used to bind to the nitrocellulose filters transferred as above, and the filters were similarly processed. The result of the variously diluted E. coli lysate on the background signals is also presented in Table 6. E. coli lysate with a dilution of 1/200 was therefore chosen to block the the first antibody used in all subsequent library screening with the polyclonal antibody.

4. *Isolation of CD9 cDNA with Antibody Probes*

Before the polyclonal antibody against CD9 antigen was available, both the NALM-6 λgt11 library and the human hepatoma λgt11 library were screened with the monoclonal antibody 50H.19. Since repeated screening failed to produce any positive clones, the ability of the monoclonal antibody to recognize the CD9 antigen in both native and denatured forms on nitrocellulose filter was tested. Platelet lysate both native and denatured was used to perform a Western blotting, using mAb 50H.19 as the detecting antibody. Figure 11 shows the result of the Western blotting. Although the mAb 50H.19 was able to recognize the denatured CD9 antigen on the nitrocellulose filter, it failed to do so once the protein became reduced (by treatment with 1% 2-mercaptoethanol). Interestingly, two other CD9 mAbs, ALB-6 and BA 2, were also found to recognize the antigen in a similar conformation-dependent manner (result not shown).

Library screening with rabbit polyclonal antibody against denatured CD9 antigen was carried out as soon as the antibody became available. Over 1×10^7 p.f.u. phage from the NALM-6 and 5×10^6 the HepG2 λgt11 libraries were screened with a 1/400 dilution of the polyclonal antibody preadsorbed with a 1/200 dilution of E. coli lysate, and the filters developed in the 3-amino-9-ethylcarbazole color reagent, which resulted in

the isolation of a positive plaque from the HepG2 library. The positive plaque was picked, and used to perform the second and third round screenings (as described in Chapter II). As represented in Figure 12, the positivity of the clone remained unchanged throughout the three rounds of screening.

5. *Rescreening of the Positive Clone with Cell Line Adsorbed Antibodies*

Since the positive clone was identified with the rabbit polyclonal antibody, and the immunogen contained, in addition to CD9 antigen, several other minor protein components, the possibility of the clone identification due to cross reactive antibody against irrelevant protein was tested by rescreening the positive clone with the CD9 polyclonal antibody preadsorbed with cells from various cell lines. The phage from the second round screening were plated out, and the antibody screening was carried out as before (with 1/400 dilution of first antibody preadsorbed with 1/200 dilution of E. coli lysate), using cell line-adsorbed polyclonal antibodies (prepared as described in Materials and Methods). Figure 13 is the result of the rescreening. Preadsorption with the strong CD9 producing NALM-6 cells greatly reduced the reactivity of the polyclonal antibody to the positive clone, whereas adsorption with HEL cells (a weak CD9 antigen producer) moderately reduced reactivity; and the reactivity was not affected at all by adsorption with Raji cells (a CD9 surface antigen negative cell line) suggesting that the clone expressed a fusion protein containing CD9 epitopes.

6. *Characterization of the CD9 Positive Clone*

a) The Positive Clone Contained a 1.3 Kb Insert

Phage DNA was isolated from the CD9 positive clone using the rapid phage DNA preparation method described previously in Chapter II. The DNA was cut to completion with the Eco RI restriction endonuclease overnight, mixed with the DNA loading buffer containing RNase, and analyzed by 0.8% agarose gel electrophoresis. Figure 14 is the picture taken after the gel was stained with ethidium bromide. A cDNA insert was clearly

seen. No insert could be detected with the uncut DNA from the same preparation (result not shown). The size of the insert was determined by plotting (with semi-logarithmic graph paper) against the size markers (λDNA cut to completion with HindIII) loaded beside the digested phage DNA, which was about 1,300 bp in length.

b) Subcloning the cDNA Insert into Plasmid Vector

In order to subclone the insert into the plasmid and M13 vector, large scale phage DNA isolation was carried out. Repeated overnight digestion with Eco RI, however, failed to generate the expected cDNA insert. The positivity of the phage used to prepare the DNA was reconfirmed by screening with the polyclonal antibody. The ability of Eco RI to cut DNA was tested by digesting plasmid pUC19 DNA, and the possibility of the existence of materials inhibitory to restriction enzyme digestion excluded by cutting with Kpn I (result not shown). To find out if the phage DNA contained any cDNA insert at all, a diagnostic digestion with KpnI and Sst I was performed. Figure 15 a is the result of the agarose gel electrophoresis analysis of the digested phage DNA. From the restriction map of λgt11 DNA we would expect the wild type DNA to generate three fragments of 1.5 kb, 2.1 kb, and 4.5 kb following digestion with Kpn I , and Sst I (Fig. 15 b). However, in addition to the phage arms, four DNA bands of 1.5 kb, 3.4 kb, 4.5 kb, and 8.0 kb were clearly seen in the lane loaded with the Kpn I and Sst I cut phage DNA. Since the expected 2.1 kb fragment was replaced by the 3.4 kb fragment it appears that the 1.3 kB insert was contained within the 3.4 kb fragment, but could not be excised with Eco RI.

To further characterize the 3.4 kb fragment containing the 1.3 kb cDNA insert, preparative agarose gel electrophoresis was performed to isolate the fragment for subcloning into the plasmid pUC19 vector previously digested with Kpn I and Sst I and dephosphorylated. Rapid plasmid DNA preparation was carried out to isolate the DNA from the white colonies, and the DNA analyzed for the presence of the 3.4 kb fragment

by digestion with Eco RI, or Eco RI plus Hind III, or Kpn I plus Sst I. Figure 16 is the result of the analysis by agarose gel electrophoresis of the cut DNAs. Surprisingly, 3 DNA bands (3.7, 1.3 kb and 1.1 kb) were seen in the lane loaded with the Eco RI cut DNA, 4 bands (2.7 kb, 1.3 kb, 1.1 kb and 1.0 kb) were seen with the Eco RI and Hind III cut DNA, while two bands were seen with the Kpn I and Sst I cut DNA, which indicated that the 1.3 kb cDNA was successfully excised from the 3.4 kb fragment contained with the plasmid DNA.

c) Subcloning the cDNA Insert into M13 Vector and DNA Sequencing

The 1.3 kb cDNA insert was isolated from the plasmid and subcloned into the M13 sequencing vector mp19 previously cut with Eco RI and dephosphorylated. The success of the subcloning was confirmed by isolating the DNA from the each white plaque, and analyzing the DNA by agarose gel electrophoresis together with the DNA isolated from the blue plaques (wild type M13 without inserts). To delete the 1.3 kb cDNA insert for the purpose of sequencing the entire cDNA by M13 universal primer, single stranded M13 DNA containing the cDNA was isolated, and used to generate the nested deletion mutants by the Dale method (see Chapter II for detail). The deletion procedure is summarized in Figure 17. The cDNA was also subcloned into the M13mp10 vector in the similar manner, and the recombinant RF DNA used to generate the deletion mutant by digestion with exonuclease III and exonuclease VII, which is summarized in Figure 18. No deletion mutant was obtained from either methods, despite repeated attempts using reagents from different sources.

The 1.3 kb insert was also subcloned into the M13mp18 RF DNA previously digested with EcoRI and dephosphorylated. Recombinant M13 were picked from the white plaques, and the single stranded DNA analyzed by agarose gel electrophoresis. Vectors containing the cDNAs were indicated by the slower mobility during the electrophoresis. Furthermore, the orientation of the insert in each M13 was analyzed by

the C-test described before. 15 µl of the single stranded DNA was randomly taken from one of the recombinant phage stock, reannealed to 15 µl of the single stranded DNA from each of the other recombinant phage stock, and the reannealed DNAs analyzed by agarose gel electrophoresis. The result of the C-test is presented in Figure 19. As suggested, the test DNA, together with the other 9 phage DNAs was complementary to 2 other phage DNAs, and hence in opposite orientation.

The sequencing reaction was carried out as described in Materials and Methods, using both the universal primers and the sequence specific primers chemically synthesized to sequence the two strand of the entire 1.3 kb cDNA insert. To increase the resolution of the sequencing gels, the reaction mixture primed by each sequencing primer was loaded five times, at least 450 bp sequence information was obtained as a result of the multiple loading. Figure 20 is the comparison between the single loading and the 5 loadings. Beside the universal primer, only two more synthetic primers were used to sequence the entire strand of 1261 bp. The sequence from one strand was converted into the complementary sequence, and compared to that of the other strand by computer for any mismatch. Figure 21 is the result of the sequence comparison. The complete match between the two strands confirmed the correctness of the base interpretation during sequence reading.

C. DISCUSSION

1. *Size Fractionation of cDNA During Library Construction*

It is extremely important to remove the shorter, incomplete cDNA molecules and linkers from the cDNA with desired sizes. Since these shorter inserts are more likely to be ligated into the vector if they are present during the ligation, resulting in a library biased towards the small molecules, and irrelevant linkers, a lot of time would be wasted in screening these recombinant phage clones. Therefore only cDNAs with sizes longer

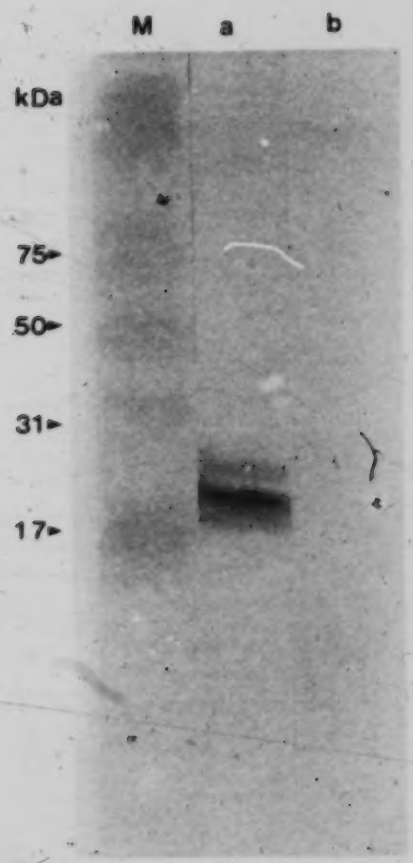


Figure 11. Analysis of the mAb 50H.19 with reduced CD9 antigen by Western blotting. Platelet lysate was mixed with sample buffer in the presence or absence of 10 mM 2-ME, boiled, and loaded onto a 12.5% polyacrylamide gel followed by separation by SDS-PAGE. The proteins were electroblotted onto a nitrocellulose filter. The filter was washed with PBS, incubated with 1/200 dilution of the mAb 50H.19 overnight at 4°C, washed with PBS again, and the antibody visualized by horseradish peroxidase conjugated goat anti-mouse second antibody (1/3000 dilution). Lane a was loaded with the lysate without 2-ME treatment, while lane b was loaded with 2-ME treated lysate. No 2-ME reduced CD9 antigen was recognized by the mAb 50H.19. Lane M was the Bio-Rad pre-stained low molecular protein size markers.

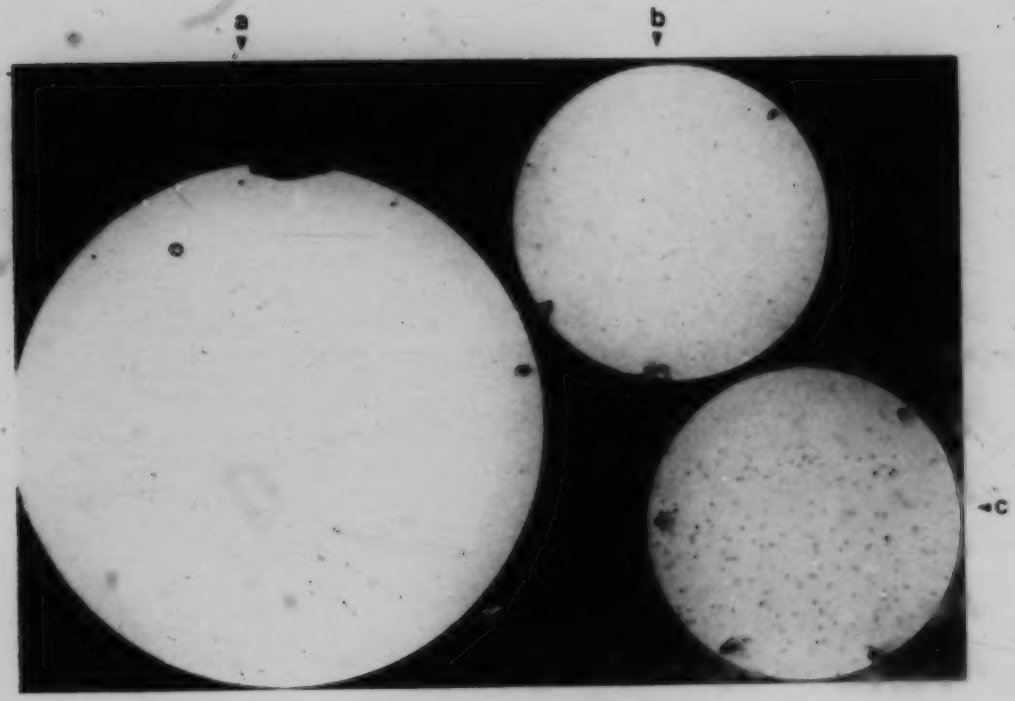


Figure 12. Isolation of the first CD9 cDNA from HepG2 λgt11 library. Phage from HepG2 λgt11 library were plated onto large LB plates (1 x 10^5 p.f.u. per plate). Positive plaques were identified by first-round screening with CD9 polyclonal antibody (Figure 12 a). The positive plaques were marked to aid recovery. Agar containing the positive clone was selected, and to ensure the isolation of the positive clone, the surrounding negative phage plaques picked as well. Phage stock was prepared, titred, and plated out (1x10^3 p.f.u. per plate) on small LB plates. Second-round screening (Figure 12 b) with the polyclonal antibody was performed, and well-isolated positive plaques selected, and stock prepared prior to a third-round screening to produce a homogeneous population of the positive phage (Figure 12 c).

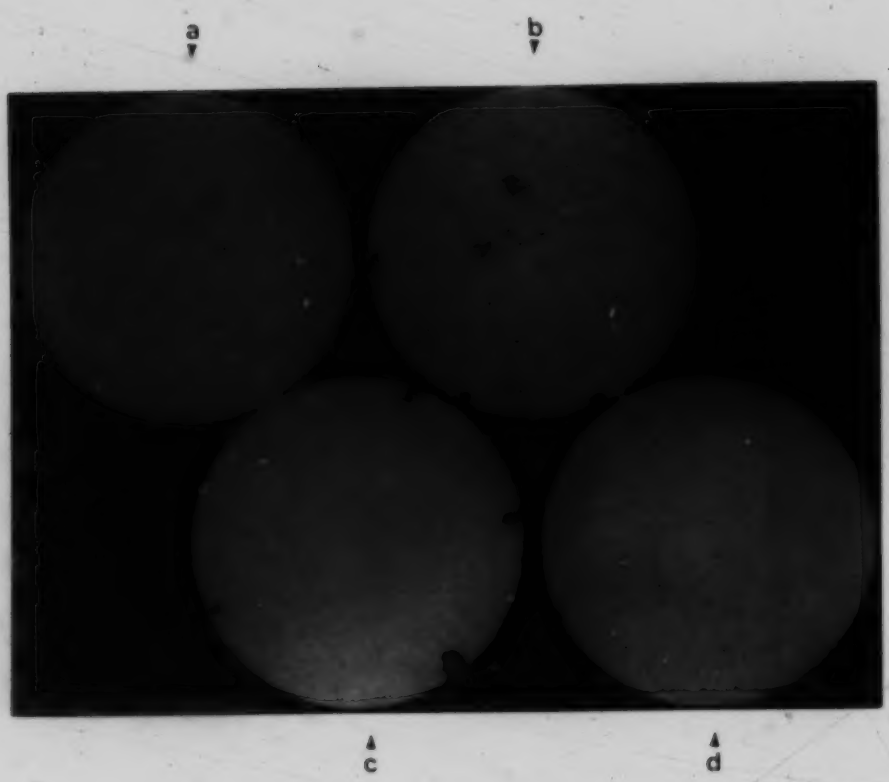


Figure 13. Rescreening of the CD9 positive clone with CD9 preadsorbed polyclonal antibodies: a test of specificity. Homogeneous CD9 positive phage were plated onto small LB plates at 500 p.f.u. per plate. Imprinted nitrocellulose from each plate was screened with either the CD9 polyclonal antibody, or with CD9 polyclonal antibody preadsorbed with cells differing in the amount of CD9 surface antigen expressed. Phage stock from the second-round screening containing a mixture of the positive and negative clones was also plated out, and screened with the straight CD9 polyclonal antibody in order to provide a reference for the distinction between positive signals, and the background (Figure 13 a). Preadsorption with CD9 negative Raji cells did not reduce the positive signals produced by the treated CD9 polyclonal antibody (Figure 13 b), whereas preadsorption with strong CD9-producing NALM-6 cells almost completely removed the positive signal Figure 13 c), and preadsorption with the low CD9-producing HEL cells resulted in partial reduction of the positive signal (Figure 13 d).

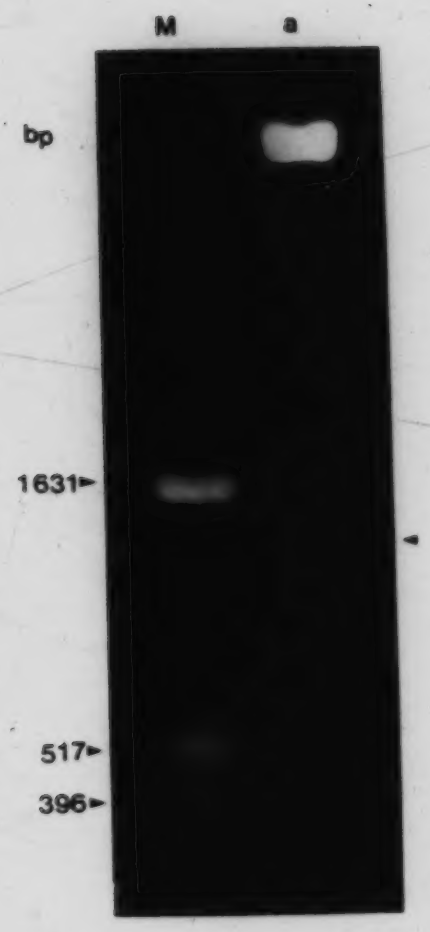


Figure 14. Analysis of the CD9 cDNA insert by agarose gel electrophoresis. Phage DNA was isolated from the CD9 positive clone, digested with Eco RI to completion, and separated on a 0.8% agarose gel by electrophoresis. The DNA bands were visualized by staining with ethidium bromide. Lane M was loaded with the DNA size markers (pBR322 DNA cut with HinF I) used to estimate the insert size, while Lane a was loaded with the CD9-positive phage DNA digested with EcoRI. A 1.3 kb insert (arrow) was detected in Lane a.

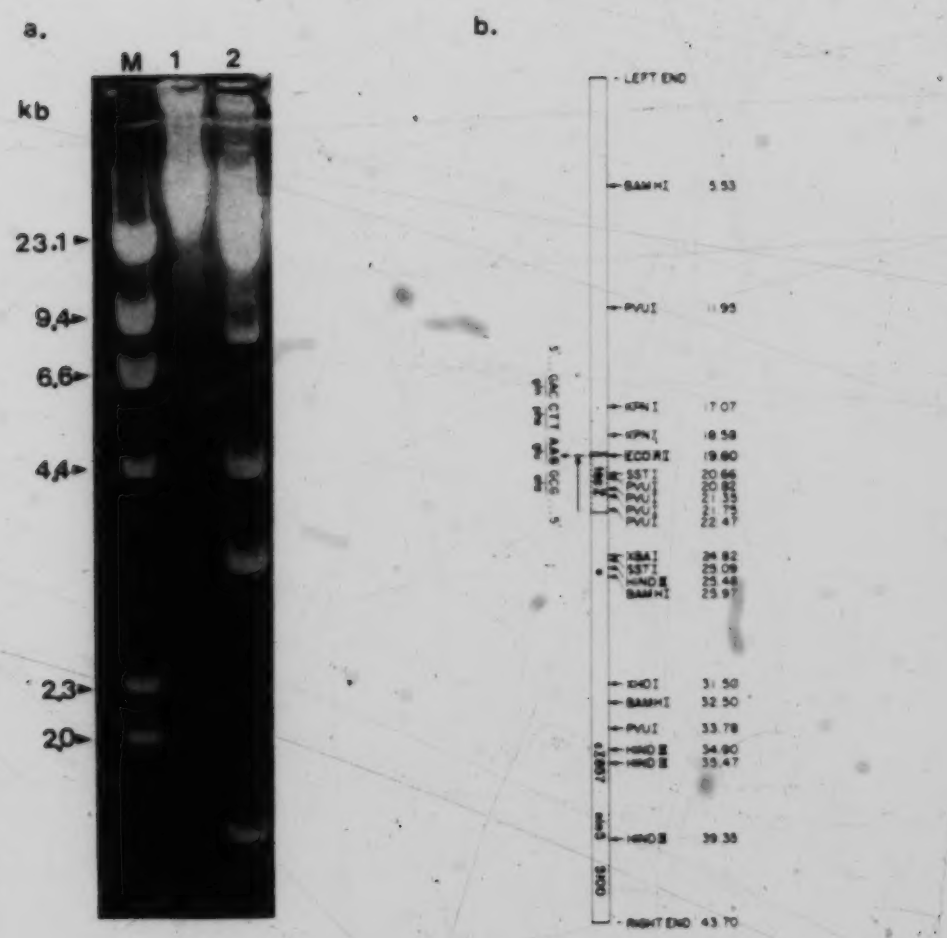


Figure 15. Analysis of the CD9 positive phage DNA by diagnostic restriction enzyme digestion. DNA was isolated from the CD9 positive phage clone, digested with restriction endonuclease to completion, and analyzed by 0.8% agarose gel electrophoresis. The DNA bands were visualized by staining with ethidium bromide (Figure 15 a). Lane M was loaded with the DNA size markers (λ DNA cut with Hind III). Lane 1 was loaded with the Eco RI cut CD9 positive phage DNA, while lane 2 was loaded with the same DNA digested with Kpn I and Ssp I. No DNA insert was detected in Lane 1, while 4 DNA fragments (1.5 kb, 3.4 kb, 4.5 kb, and 8.0 kb) were detected in Lane 2 (in addition to the lambda arms). The expected 2.1 kb fragment was replaced by the 3.4 kb fragment.

Figure 15 b is the restriction map of λgt11 DNA. Digestion of the wild-type DNA with Kpn I plus Sst I would generate three fragments, 1.5 kb, 2.1 kb, and 4.5 kb, excluding the phage DNA arms.

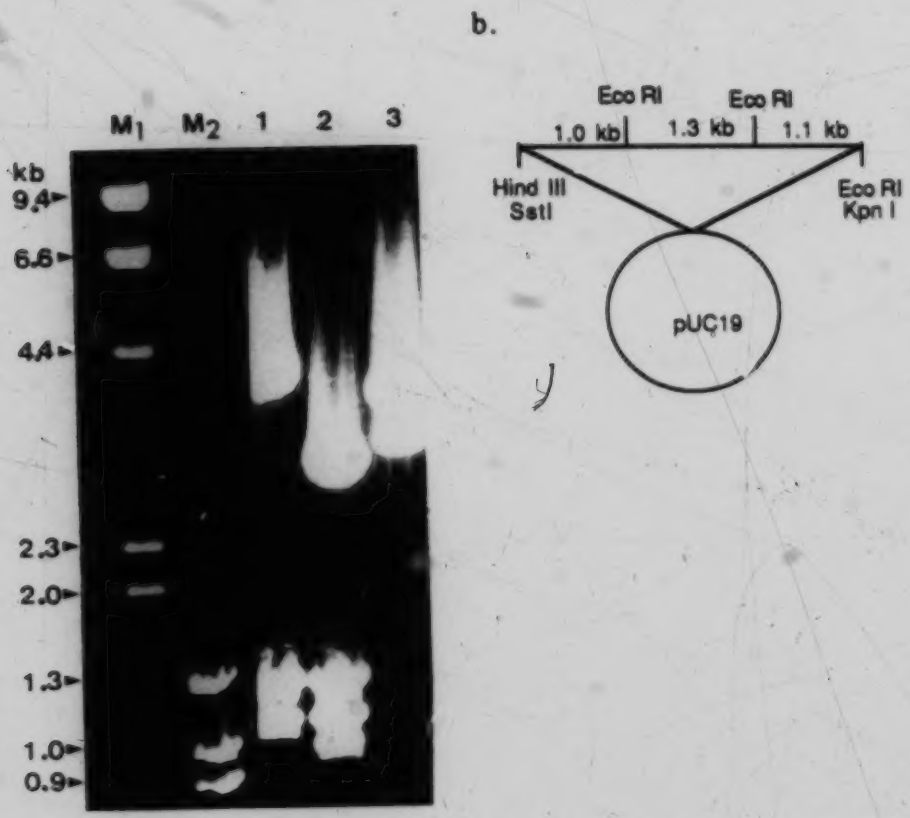


Figure 16. Analysis of the 3.4 kb fragment containing plasmid by diagnostic restriction enzyme digestion. DNA was isolated from the plasmid containing the 3.4 kb fragment, cut with restriction endonucleases to completion, and analyzed by 0.8% agarose gel electrophoresis. The DNA bands were visualized by staining with ethidium bromide (Figure 16 a). Lane M_1 was loaded with the DNA size markers λ DNA cut with Hind III, while Lane M_2 was loaded with the DNA size markers obtained from Dr. James Friesen's laboratory. Lane 1 was loaded with the DNA digested with Eco RI, Lane 2 was loaded with the DNA digested with DNA Eco RI plus Hind III, while Lane 3 with DNA digested with Sst I plus Kpn I. Three DNA bands with sizes of 3.7 kb, 1.3 kb, and 1.1 kb each were seen in Lane 1, four bands with sizes of 2.7 kb, 1.3 kb, 1.1 kb, and 1.0 kb were seen in Lane 2, while two bands of 3.4 kb and 2.7 kb were seen in Lane 3.

Figure 16 b is the restriction map of the recombinant plasmid carrying the 3.4 kb fragment.

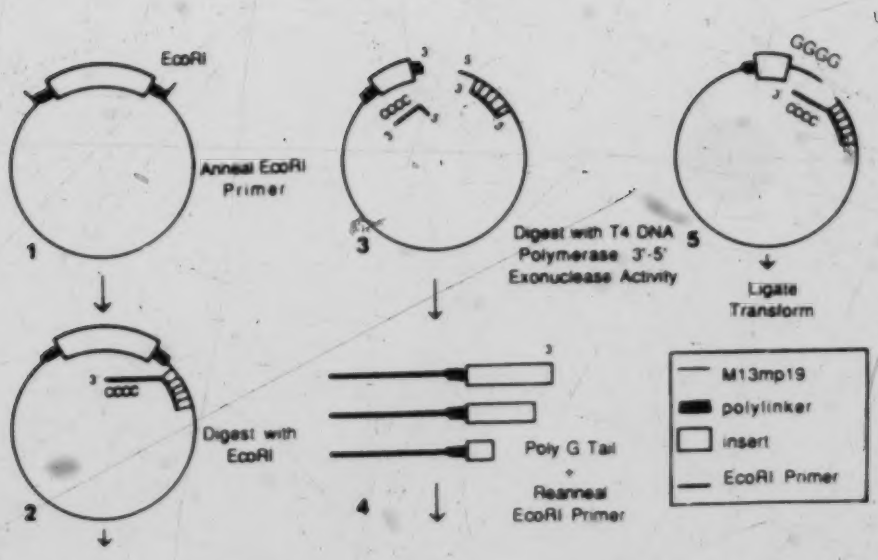


Figure 17. Generation of deletion mutants by Dale deletion. Eco RI primer was annealed to the single stranded M13mp19 carrying the 1.3 kb cDNA insert. The double stranded Eco RI sequence was then cleaved with Eco RI, which created a single stranded 3' end in the DNA immediately before the insert. The insert was degraded to various distances with T4 DNA polymerase which only removes nucleotides from 3' to 5' direction. The reaction was terminated by heating to 65°C. Poly-dG tail was added to the remaining insert, followed by reannealing back the Eco RI primer. The single stranded DNA was rejoined through hybridization between the poly-dG tail added to the insert and the poly-dC sequence on the primer, and the nick was closed by ligase in the presence of ATP. The ligated plasmids were then used to transform the competent bacteria in order to select the deletion mutants.

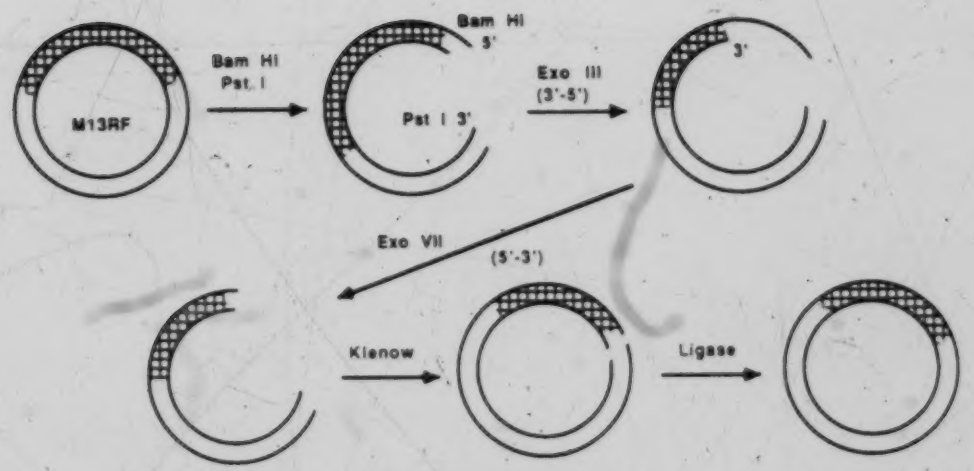


Figure 18. Generation of deletion mutants by exonucleases. Double stranded RF M13mp10 DNA carrying the 1.3 kb cDNA insert was digested with Bam HI which created a 3' overhang on the M13 DNA, and with Pst I which created a 5' overhang immediately before the insert. One strand of the insert was degraded to various distances from 3' to 5' by exonuclease III which only removes nucleotides from the 5' overhang. The single stranded DNA was trimmed with exonuclease VII, the DNA was blunt-ended with Klenow fragment, and ligated. The deleted M13 RF DNAs were then used to transform the competent bacteria in order to select the deletion mutants.

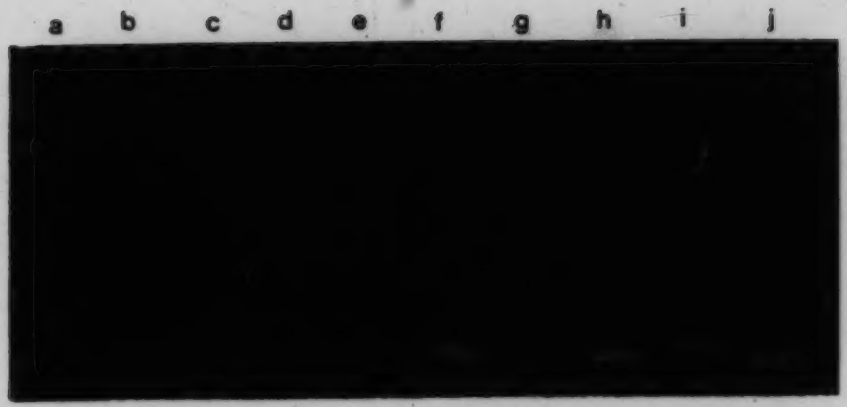


Figure 19. Determination of the insert orientation by C-test. Single stranded recombinant M13 DNA containing the cDNA insert was chosen at random and reannealed to each of the 10 other single stranded sample M13 DNA. The reannealed DNAs were analyzed by a 0.8% agarose gel by electrophoresis followed by staining with ethidium bromide. Compared to the DNAs in the other lanes, the reannealed DNAs in Lane d and Lane g moved much slower, indicating that their molecular weights were increased as a result of the hybridization between the randomly chosen M13 DNA and the two sample DNAs.

Figure 20. Extension of sequencing resolution by multiple loading. Freshly isolated single stranded M13mp19 DNA containing the 1.3 kb cDNA insert was sequenced by dideoxy chain termination method with universal primer. After denaturation by boiling followed by quick chilling on ice, the newly synthesized DNAs were separated on a sequencing gel, and the gel was then fixed, dried, and used to obtain an autoradiography. The electrophoresis was terminated when the bromphenol blue dye reached the bottom of the gel (Track a). Alternatively, each time the blue dye reached the bottom of the gel, another loading was performed using the same sequencing reaction mixture, and the electrophoresis terminated when the blue dye from the fifth loading had reached the bottom (Track b to f). Dense DNA bands representing large synthesized DNA molecules were seen in each track, which were better separated as a result of the multiple loading. At least 450 bp can be read from one sequencing reaction.

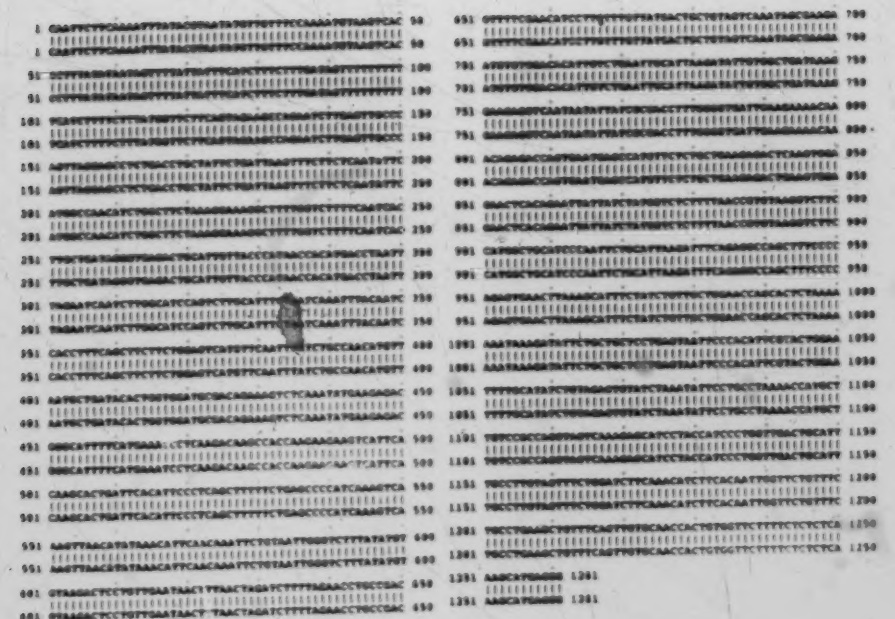


Figure 21. Comparison of the DNA sequences from two strands of the cDNA insert. Sequencing of the 1.3 kb cDNA insert was accomplished by using both universal primer and sequence specific primers. Excluding the universal primers, only four sequence specific primers were chemically synthesized in order to sequence the entire cDNA on both strands. The DNA sequence from one strand was converted to the complementary sequence, which was matched to the sequence from the other strand. The complete match between the two sequence confirmed the correctness of the base-assignment made when manually interpreting the autoradiogram films. The distance of the individual nucleotide from the 5' end of the cDNA is indicated by the numbers on both ends of the each sequence segment.

than 800 bp were recovered during size selection by agarose gel electrophoresis. The determination of the cut off value for the desired cDNA is based on the following generally accepted calculation:

$$\text{minimal length of CD9 mRNA} = \text{length of the coding region + non-coding region}$$

$$= \frac{\text{20,500 (MW of CD9 precursor)}}{\text{110 (MW of amino acid)}} + \text{200 (bases)}$$

$$= \text{860 (bases)}$$

2. *Optimizing the Screening Conditions*

At the time the initial screening was carried out, there were only a limited number of publications on gene cloning from λgt11 libraries with antibodies. It was therefore necessary to determine which of the published screening methods would be the best for screening with antibody. Although the horseradish peroxidase-conjugated second antibody gave a weaker signal compared to the ^{125}I-protein A, it was chosen for library screening because of its higher specificity. In addition, the positive clone could be visualized without overnight, or longer exposure. No radioactive material was needed to perform the assay. Furthermore, the weakness of the signal could be enhanced by replacing the Bio-Rad color reagent (which produces a purple color) with the 3-amino-9-ethylcarbazole (which gives rise to a brown color). Gelatin blocking was favored over blocking by fetal calf serum because the former produced lower background. Despite the high sensitivity of the ^{125}I-protein A, the lower degree of specificity made it a less favored candidate. In order to avoid wasting time in characterizing numerous false positive clones during large scale screening, the specificity of the detection method employed is more important than its sensitivity. The importance of specificity was best illustrated by the 98 false positive plaques picked up during the initial screening of the

NALM-6 library with mAb 50H.19 when ^{125}I-protein A was used to detect the bound monoclonal antibody.

The specificity of the screening was also affected by the polyclonal antibody. Since the antibody was raised in animals where sensitization by E. coli protein was the rule, the antibody obtained was very likely to contain cross reactivity to E. coli proteins. To prevent false positivity due to such cross reactivity, it would be necessary to remove the cross reactive antibodies which were directed against E. coli proteins. This was achieved by diluting the polyclonal antibody (the titre of cross reactive antibodies was much lower than that of the CD9 antibody, hence more sensitive to dilution), and by blocking with E. coli proteins. Since antibody dilution below 1/400 would cause loss of the background signals (needed to determine if the assay worked or not), and could possibly reduce the signal, an antibody dilution of 1/400 was chosen. On the other hand, since the E. coli lysate diluted below 1/200 seemed to lose the blocking effect, a 1/200 dilution was chosen. Although higher concentration of E. coli lysate seemed to result in better blocking, the use of higher amounts of E. coli lysate would be costly, and the excess amount of E. coli protein might interfere non-specifically with antibody binding.

3. *The Importance of Polyclonal Antibody in λgt11 Library Screening*

Before the polyclonal antibody was available, the NALM-6 library was screened with the mAb 50H.19. At that time, the general assumption was that as long as the monoclonal antibody was able to recognize the antigen fixed on a nitrocellulose filter, it could be used to screen the λgt11 library. Although mAb 50H.19 did recognize the SDS-denatured CD9 antigen in a Western blot, repeated screening of both the NALM-6 and the HepG2 libraries failed to pick up any positive plaques. This was likely due to the fact that mAb 50H.19 recognized a conformationally determined epitope. Since E. coli does not have the machinery for post-translational protein modifications such as glycosylation or acylation, and the eukaryotic proteins were made in many instances as

insoluble aggregates of denatured protein (Wetael and Goeddel, 1983), proper folding of the expressed protein was a very unlikely event. Furthermore, the secondary structure of the foreign protein would also be affected by the β-galactosidase, which was part of the fusion protein. Co-incidentally, when screening with mAb 50H.19 was about to be finished, a report by Timmins et al. (1985) was published, which stressed the importance of raising polyclonal antibody against denatured antigen in gene cloning from λgt11 library. Although the same difficulty was encountered by these authors in gene isolation with a monoclonal antibody, the problem was successfully solved by rescreening the original library with the polyclonal antibody.

4. *Frequency of the CD9 cDNA in λgt11 Libraries*

If one assumes that there are 15 molecules of mRNAs (representing a low abundance protein such as CD9) in a total of 360,000 mRNAs molecules from a CD9 producing cell (Alberts et al., 1983), every 24,000 mRNAs would contain a CD9 mRNA. A cDNA library 6 times the size of 24,000 recombinant clones (144,000 clones) should have at least one CD9 encoding cDNA, considering that the ribosome may use any of the three reading frames, and either of the two strands during protein translation. However, this is only the best scenario, since the cDNAs synthesized during library construction are often shorter than the mRNA template because of the early termination of the first and/or second strand synthesis. To obtain a library with full length copy of the mRNA encoding a particular protein, a larger library size is always needed. It is particularly true when the protein product of the cDNA rather than the cDNA itself is used to select the specific cDNA clone. Deletion of any region involved in forming the epitope necessary for antibody recognition would result in failure of the antibody screening. The failure to obtain a longer length cDNA copy of the CD9 mRNA may explain the inability to isolate a CD9 positive clone from the NALM-6 λgt11 library with the polyclonal antibody, since the library had an original packaging titre of 4×10^5

p.f.u., less than 3 times the minimal library size requirement. This concept is further supported by the successful isolation of the shorter 0.7 kb cDNA from the NALM-6 library once the cDNA probe was used to select the CD9 positive clones. The HepG2 library on the other hand has an original packaging titre of 1.5×10^6, and is therefore more likely to contain a longer length CD9 cDNA insert. However, HepG2 expresses a low level of CD9 antigen, which may account for the detection of only one positive plaque in 4×10^6 p.f.u. phage.

5. *Possible Causes for the Loss of Eco RI Site*

The problem encountered in excising the cDNA insert from the phage DNA is intriguing. That the problem may lie in the restriction enzyme was excluded by the control digestion of plasmid DNA, and the purity of the phage DNA confirmed by digestion with Kpn I. Therefore, it was initially concluded that the cDNA insert may have been lost from the phage DNA, which is a recognized problem in cloning with phage vector. Diagnostic digestion with both Kpn I and Sst I revealed that although the insert could not be cut out with Eco RI, it was still present in the phage vector (Figure 15 a). As indicated by Figure 15 b, three bands (1.5 kb, 2.1 kb, and 4.5 kb) would be expected if the wild type phage DNA was digested with both Kpn I and Sst I. However, when the recombinant phage DNA was similarly digested, the 2.1 kb fragment disappeared, and was replaced with a 3.4 kb fragment, which indicated that a 1.3 kb cDNA was present within the fragment, the same cDNA insert seen when the clone was first isolated and analyzed. Interestingly, an 8.0 kb fragment was always present when the phage DNA was cleaved with both the Kpn I and the Sst I, which could not be explained by the published restriction map. This may either due to the omission by the author (because the site was far away from the cloning site?) or due to a mutation in the vector which generated a new restriction site.

Surprisingly, when the 3.4 kb fragment containing the 1.3 kb cDNA insert was subcloned into the pUC19 plasmid vector, the cDNA could now be excised from the flanking phage DNA. This was clearly shown in Figure 16 a. As indicated by Figure 16 b, if the Eco RI sites on both side of the 1.3 insert were lost, digestion with Eco RI would only linearize the entire DNA, resulting in a single DNA band (6.1 kb), while digestion with Eco RI and Hind III would only generate two bands (3.4 kb and 2.7 kb) like the control digestion with Kpn I and Sst I.

The most common cause of the loss of the cloning site is that site modification occurred during cloning. After the restriction site was opened with the restriction enzyme, the contaminating nuclease would cleave off some bases from the 'sticky ends'. Although this may not interfere with the subsequent ligation, the restriction site would be permanently lost once the modified 'sticky ends' were religated. However, the fact that the 1.3 kb insert was recovered from the 3.4 kb fragment with Eco RI digestion would argue against this possibility. The other probable cause could be steric hindrance from the phage DNA blocking the Eco RI sites which was removed once the rest of the phage DNA was separated from the 3.4 kb fragment. However, the same phage DNA was present when the 1.3 kb insert was initially successfully excised from the entire phage DNA. Furthermore, it is hard to imagine how both Eco RI sites could be blocked at the same time by the phage DNA. Since the two Eco RI sites were initially present, and became lost only during the subsequent phage growth in E. coli, it is possible that the Eco RI sites were eventually methylated by the host bacterium. It is still difficult, however, to explain why the methylated sites were again demethylated when the 3.4 kb fragment was subcloned into the plasmid and propagated, unless the intact phage DNA could up-regulate the E. coli Eco RI methylase while the plasmid could not. Similar factors involved in preventing the recovery of the insert from the λgt11 could also be implicated in the failure to generate deletion mutants.

6. *Multiple Loading Extended the Resolution During Sequencing*

A major limiting factor in DNA sequencing using the dideoxy chain terminating method is the poor resolution of the larger fragments, since these large fragments run more slowly during the separation, and become densely stacked. Common 'tricks' used to separate these fragments were to run longer gels, or to double or triple load the DNA sample. Also, gradient gels were introduced that gave a better separation of the larger fragments. With these methods, up to 350 bp DNA could be sequenced with each sequencing primer. However, if the template was very 'clean', and the sequencing reaction was efficient, still larger fragments could be synthesized from a single primer. These larger fragments, once separated, could further extend the resolution so that more base pairs could be read from each sequencing reaction. This became an important factor to consider when sequence specific primer was used to prime the sequencing, especially when the oligonucleotide synthesis facility was not handy. Although convenient DNA synthesis facilities were available to carry out the CD9 cDNA sequencing, extending the sequencing resolution would still speed up the sequencing procedure, and would reduce the cost as well. Attempts were therefore made to extend the resolution by improving the quality of the sequencing reaction using freshly isolated template, adjusting the composition of the dNTPs, and by multiple loading of the synthesized DNA fragments. At least 100 more bp information was obtained as the result of the multiple loading, which enabled the entire strand to be sequenced with only two sequence specific primers (without the multiple loading, one more primer would be needed to cover the 1.3 kb insert).

CHAPTER V

ANALYSIS OF THE cDNA SEQUENCE

A. INTRODUCTION

Once the DNA sequence from the 1.3 kb insert was obtained, the immediate questions to be addressed included whether the DNA encoded any protein at all, if so whether the encoded protein was CD9 antigen, and whether it bore homology to other sequences. The answers to these questions were provided by the computer analysis of the DNA sequence, which is presented in the following discussion.

B. RESULTS AND DISCUSSION

1. *The cDNA May Encode a Large Protein*

Since a cDNA can be translated into protein in three reading frames from both strands, it is necessary to determine the correct reading frame. Fortunately, the improper reading frame is frequently interrupted by stop codons, whereas the reading frame used in protein translation (open reading frame, or ORF) predicts a continuous amino acid sequence. This provides a convenient way to locate the correct coding frame. Figure 22 shows all the six possible reading frames of the cDNA plotted by the computer. It is clear that the second reading frame is the correct frame to use for analyzing the encoded protein of the cDNA. The long ORF found in this frame is 880 bp in length and has the potential to encode a 32 kDa protein.

Analysis of the strand that possessed the long ORF revealed the existence of a potential poly-adenylation signal (poly A signal) AATAAA about 300 bp downstream from the protein translation stop codon, as indicated in Figure 22 b. Since the poly A signal is usually followed, about 10 to 30 nucleotides downstream, by a string of A residues (or poly A tail), the fact that no such poly A tail could be seen even 65

nucleotides downstream would seem to suggest that this potential poly A signal may not be utilized for poly-adenylation.

2. *Protein Translation May Start at the Fifth Start Codon*

Figure 23 is the amino acid sequence predicted from the long open reading frame. As indicated, there are eight translation start codons within the entire ORF. It is important therefore to decide which start codon is actually used by the ribosomes during translation.

An important difference between prokaryotic and eukaryotic protein translation is that the translation is initiated by binding of the ribosomes to a ribosomal binding site (Shine-Dalgarno sequence) in prokaryotic systems, whereas in eukaryotic systems the translation is initiated with binding of the cap site by the ribosome which moves downstream until it meets a start codon in the right context (Lewin 1985). By comparison among 699 vertebrate mRNAs, Kozak (1987) had identified some interesting features of functional eukaryotic start codons: the 6 conserved nucleotides (GCCACC or GCCGCC) immediately before the ATG start codon, the conserved nucleotide G immediately after the start codon, and the periodical occurrence of nucleotide G in position -3, -6, and -9. The significance of such conserved sequence would be to generate the right context in which the start codon serves to stop the downstream movement of the ribosome, since although most protein translations start at the first ATG, there are a number of exceptions. Whether a particular ATG would be used as the start codon depends on the surrounding DNA sequences, those with neighboring sequence that have the highest homology with the consensus nucleotides (Kozak sequence) are suggested to most likely serve as the start codon (Kozak, 1987). A Kozak sequence was found at the fifth ATG codon of the long open reading frame. Figure 24 is the comparison for the fifth ATG with the consensus functional start codon.

7 nucleotides completely matched the 8 consensus sequence. In fact the fifth ATG is even closer to the consensus sequence than the start codon of human proinsulin.

3. *Translated Protein from the Fifth ATG is Very Likely to be the CD9 Antigen*

That the fifth ATG is probably may be used as the start codon is further supported by the protein product translated from the start site which almost completely matches the structural data obtained from CD9 peptide mapping.

Figure 25 is the secondary structure of the predicted CD9 protein. It has a hydrophilic carboxyl-terminal, suggesting that if the protein is a membrane protein (which is supported by its homology with immunoglobulin superfamily), it should be a non-integral membrane protein. This confirms the earlier study by Newman et al. (1982) which established that CD9 antigen is a non-integral membrane protein. A stretch of hydrophobic amino acids representing a putative signal sequence for membrane-bound or secreted proteins was not found. In fact the amino terminus of the protein following the Kozak sequence is hydrophilic.

More compelling evidence that supports the conclusion that the isolated cDNA encodes CD9 protein comes from comparison of the predicted peptide cleavage sites on CD9 with the actual cleavage sites confirmed by peptide mapping experiments (Seehafer et al., 1988b). S. aureus V8 protease is known to cleave peptides at the carboxyl-terminal (C-terminal) side of glutamic acid, cyanogen bromide cleaves at the C-terminal side of methionine, formic acid cleaves in between aspartic acid and proline, while papain cleaves lysine or arginine on either side. Figure 26 a is comparison of the predicted cleavage pattern with the established pattern. Except for the first V8 fragment, all the other cleavage fragments completely match one other.

When the molecular weight of each peptide fragment is calculated from the amino acid composition, and compared to the molecular weight estimated by SDS-PAGE, the similarity provides persuasive evidence that the cDNA may indeed encode CD9. Table 7

is the summary of the predicted molecular weights of the entire CD9 precursor as well as the various peptide cleavage fragments, in comparison to the molecular weights established from peptide mapping using CD9 protein. With the exception of the first V8 fragment, a complete match between the predicted data and the established data is obtained

The discrepancy observed with the first V8 fragment is interesting. From the cDNA sequence we would expect the peptide to be 1.1 kDa. However, a 4 kDa fragment was deduced by the presence of 7 kDa and 11 kDa fragments from the proteolysis of a 22 kDa mature protein, and was in fact visualized by digestion of CD9 from SKOSC cells (Seehafer et al., 1988b). One possible explanation for the discrepancy might be that the fragment is extensively modified post-translationally, for example by glycosylation while another is that there is more than one CD9 gene

Finally, the site of N-linked glycosylation predicted by the deduced amino acid sequence also matched that determined by peptide mapping, as shown in Figure 26b. The N-linked carbohydrate of the CD9 antigen was located within the 7 kDa V8 fragment, while the location of the O-linked carbohydrate site is unclear. The predicted N-linked carbohydrate attachment site (N X S) is also found in that 7 kDa V8 fragment three amino acids downstream from the V8 cleavage site, while the O-linked carbohydrate could be anywhere along the peptide, since the O-linked carbohydrate attachment site is less specific.

4. *The CD9 Antigen May Belong to Immunoglobulin Superfamily*

Search through the Bionet as well as the Genebank database at the Department of Medical Genetics, University of Toronto and at the Ontario Cancer Institute (Toronto, Ontario) confirmed that the cDNA isolated is unique (the last search of the Bionet database was carried out on November 23, 1988). Very limited sequence homology was observed between the CD9 sequence and some other proteins such as kinase related

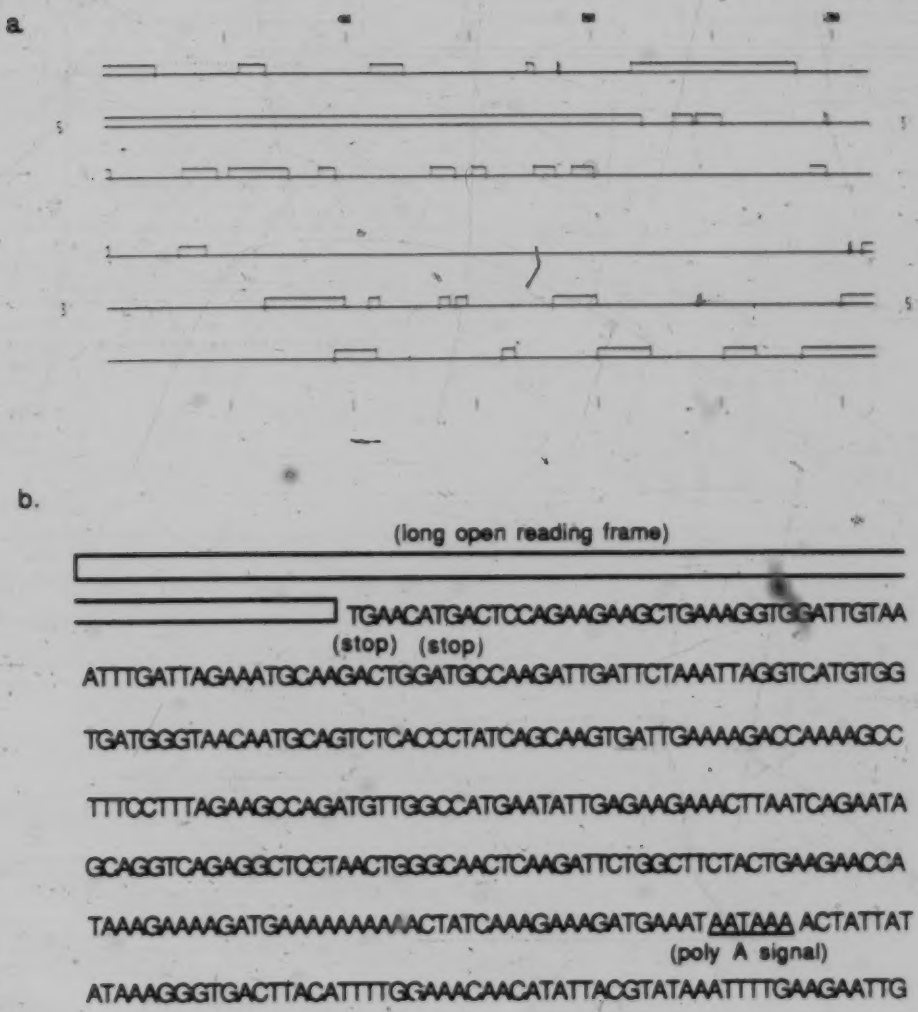


Figure 22. Long open reading frame and poly-adenylation signal possessed by the cDNA. All six reading frames of the cDNA sequence were plotted by a computer program. The boxes represent a stretch of amino acids with no protein translation stop codons in between (open reading frame, or ORF), and the vertical lines represent the distance in base pairs from the 5' end of the cDNA. The second reading frame on the first cDNA strand possesses an open reading frame (ORF) of 880 bp in length (Figure 22 a). The same DNA strand has a possible poly-adenylation signal, AATAAAA as underlined, about 300 bp downstream from the end of this long ORF (Figure 22 b).

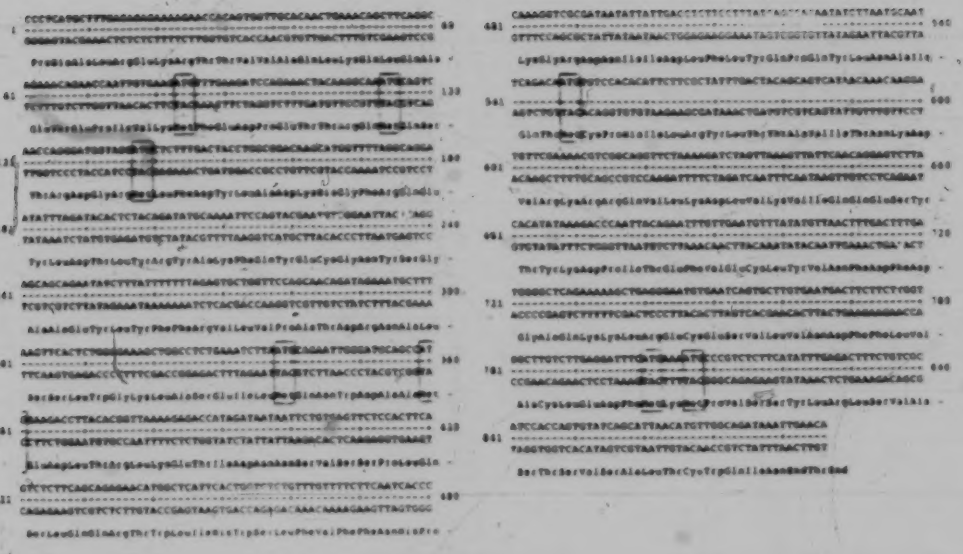


Figure 23. Amino acid sequence of CD9 predicted from the cDNA nucleotide sequence. The long open reading frame of the cDNA starts at the second base on the first cDNA strand and continues to the 880th base when a protein translation stop codon TGA is reached. The distance of the individual nucleotide from the 5' end of the cDNA is indicated by the numbers on both ends of each sequence segment. There are eight possible ATG start codons within the entire ORF, as indicated by boxes.

```
                          A
CONSENSUS -  G N N G C C G C C  ATG  G -
CD9       -  G A T G C A G C C  ATG  G -
HU INSULIN -  C C C G C A G C C  ATG  G -
```

Figure 24. Comparison of the CD9 start codon with the Kozak sequence. The nucleotide sequence surrounding the fifth ATG in the long reading frame of CD9 cDNA is compared to the consensus sequence surrounding the functional eukaryotic start codon (Kozak sequence). As underlined,there are three important features of the functional eukaryotic start codons: the consensus GCCACC or GCCGCC immediately before the ATG, the consensus G immediately after the ATG, and the periodic occurrence of G in position -3, -6, and -9. Except for the A at position -4, all the other nucleotides surrounding the fifth ATG in CD9 cDNA match the Kozak sequence. The surrounding sequence of the fifth ATG of the CD9 cDNA has a higher degree homology to Kozak sequence than that of human proinsulin.

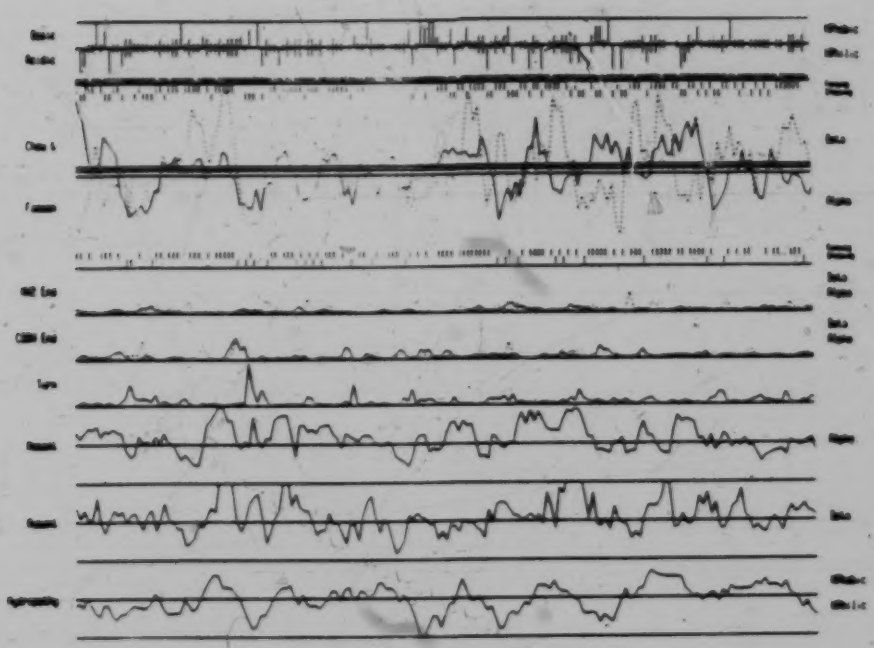


Figure 25. Secondary structure of the predicted CD9 peptide. The amino acid sequence of the CD9 protein predicted from the cDNA sequence was analyzed by a computer program, and the secondary structure of the protein was determined by the biochemical properties of the individual amino acids. The protein contains 7 hydrophilic regions including a hydrophilic region at each terminus.

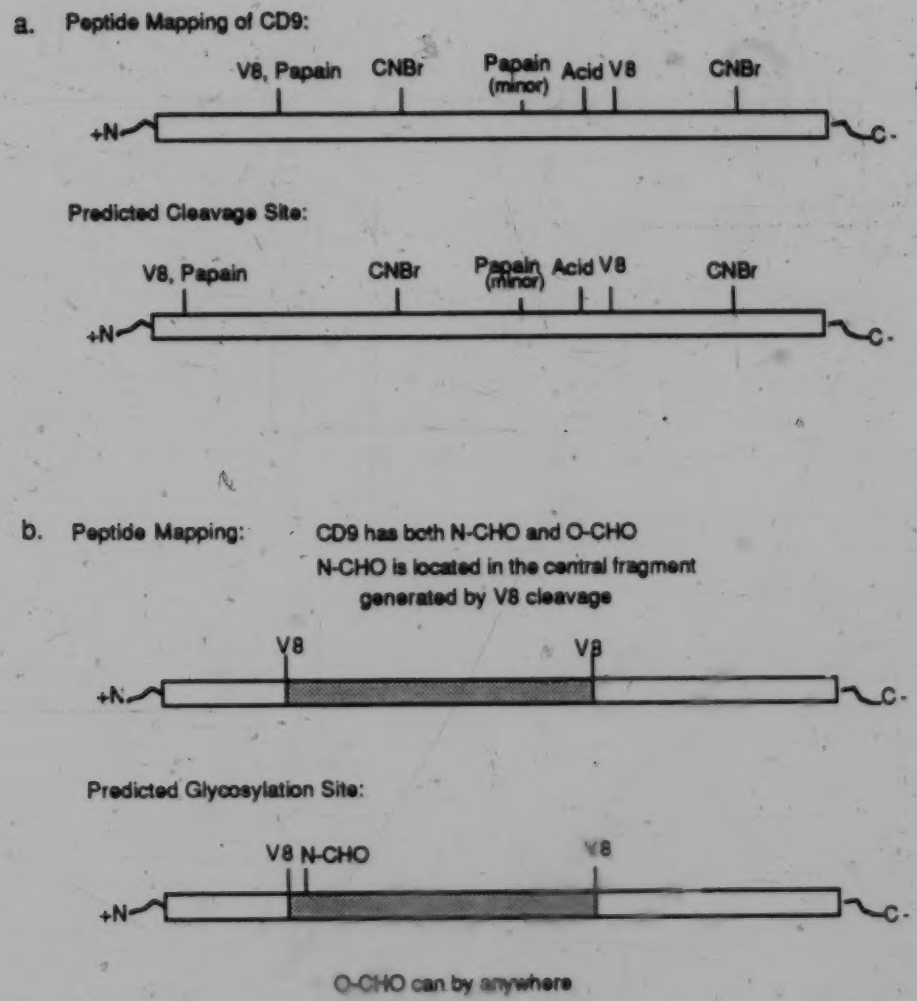


Figure 26. Comparison of the predicted CD9 proteolytic pattern with that determined by peptide mapping. Amino acid sequence of the CD9 deduced from the cDNA nucleotide sequence was used to predict the proteolytic pattern of the native protein, based on the known recognition sequence of the cyanogen bromide (CNBr), S. aureus V8 protease (V8), papain, and formic acid (acid). The predicted proteolytic sites were then compared to those determined by peptide mapping using the native CD9 protein (Figure 26 a). Except for the first V8, and papain cleavage sites, the predicted sites matched those deduced from proteolysis studies very closely.

Figure 26 b is the comparison of predicted glycosylation sites with those determined by peptide mapping.

TABLE 7

COMPARISON BETWEEN THE PROTEOLYTIC FRAGMENTS DETERMINED BY PEPTIDE MAPPING AND THE PEPTIDES PREDICTED BY THE cDNA

Proteolytic Reagent	Molecular Weight of Peptide Fragment (kDa)	
	Peptide Mapped	Peptide Predicted
CD9 Protein Precursor	20.5	20.42727
V8	4	1.11627
	7	7.04706
	11/12	12.26394
Acid	8	7.86297
	14	12.56431
Papain	4	1.02319
	10	10.47295
	18	19.44012
CNBr	3	2.60091
	8	7.64860
	11	9.97943

The molecular weights of the mapped peptides were determined by two dimensional SDS-PAGE analysis of the proteolysis cleavage products.

The molecular weights of the predicted peptides were calculated on the basis of the known proteolytic cleavage sites.

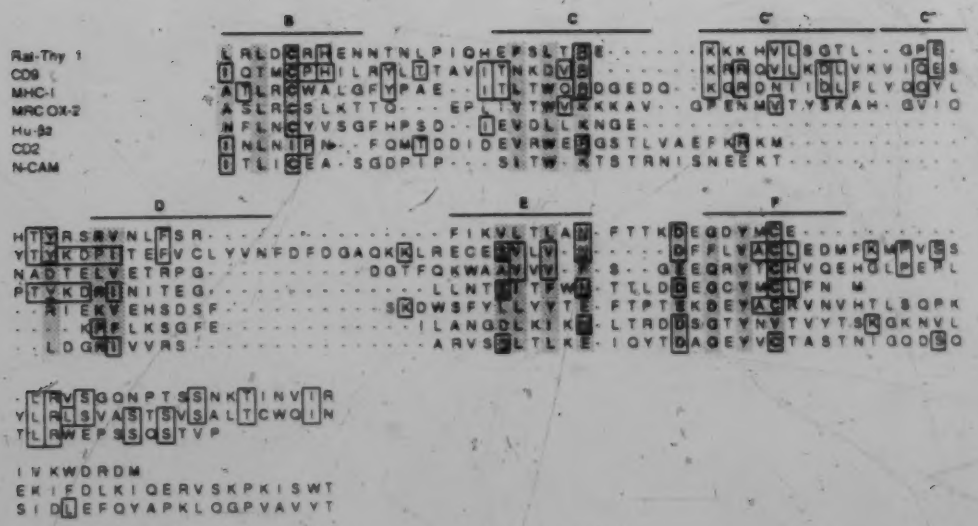


Figure 27. The cDNA has homology with the immunoglobulin supergene family. The predicted CD9 protein sequence was compared to established protein sequences of the members of the immunoglobulin supergene family: rat thy-1 antigen, MHC-I antigen, MRC OX-2 antigen, human β-2 microglobulin, CD2, and N-CAM. Gaps (dotted lines) have to be introduced (Williams, 1987) into these sequences in order to align critical conserved residues (shadowed) which determine structural features of the immunoglobulin family including disulfide bonding. Gaps were also introduced into the predicted CD9 sequence to improve alignment. Very highly conserved critical residues are shown in bold type. Relatively conserved critical residues are underlined. Two out of 3 critical residues are identical in strand B, 1 out of 2 in strand D, 2 out of 2 in stand E, and 3 out of 4 in strand F of CD9. Four other residues of the CD9 protein matched the relatively, but not critically conserved residues (1 in strand C, 1 in D, and 2 in E). 17 out of 114 residues (15%) from the CD9 proteins are identical to those of rat Thy-1 while 18 out of 114 residues (16%) are identical to those of MHC-I.

transforming protein src, H-2 class II and class I histocompatability antigen, kinase related transforming protein ras, and myosin heavy chain. The significance of these homologies was difficult to interpret. Less regionalized homologies, however, were found when the CD9 sequence was compared to the members from the immunoglobulin superfamily, rat Thy-1 antigen, MHC-I antigen, MRC OX-2 antigen, human β_2 microglobulin, CD2, and N-CAM. Figure 27 is the result of the sequence comparison. Gaps were introduced into CD9 protein in order to align it to the highly conserved critical residues among members of the family (bold letters) within the B, C, D, E and F strands of the β fold described by Williams (1987). According to Dayhoff's scoring matrix for amino acid replacement (Dayhoff et al., 1982), certain amino acids are interchangeable with other amino acids when comparing sequence homology because they share similar biochemical characteristics such as charge and hydrophobicity. The alignment resulted in a match of 2 out of 3 critical residues within strand B, 1 out of 2 within D, 2 out of 2 within E, and 3 out 4 within F. Furthermore, four residues in CD9 protein matched the relatively conserved (>40%) amino acids (underlined) among members of the family (1 in strand C, 1 in D, and 2 in E). More importantly, two cysteine residues in CD9 completely matched the highly shared cysteines, which are considered essential for maintaining the folding of the molecules belonging to the family. Finally, homologies between the CD9 protein and certain members of the immunoglobulin family were also found in non-critical residues. The overall sequence identity between the rat-Thy 1 antigen and the CD9 protein is 15%, and that between MHC-I and CD9 is 16%.

The homology found between the CD9 antigen and the immunoglobulin superfamily is very significant. All the members from the family are located on the plasma membrane, which suggested that the protein encoded by the cloned cDNA is a membrane protein as well. More importantly, the immunoglobulin superfamily is now

considered to originate from molecules that first evolved to mediate intercellular interactions (Williams, 1987). The neural cell adhesion molecule (N-CAM) is a confirmed member of the family, as well as the receptor for platelet derived growth factor (PDGF), which is *a bona fide* signal transducing molecule. The homology between CD9 and Thy-1 molecule is most interesting, since the two molecules are not only structurally similar, both are glycosylated non-integral membrane proteins that are attached to plasma membrane through fatty acids, the two molecules are also functionally related, both are involved in cell activation events. This provides strong support for the representation of the CD9 gene by the isolated cDNA clone. The lower homology exhibited by CD9 antigen to other members of the immunoglobulin superfamily suggests that CD9 may have segregated from the family at an early stage during evolution. While the remaining members of the family retain the high degree of homology in order to interact with each other through homophilic interaction, CD9 was nc longer under such functional restraint, since it had evolved to help the receptors to bind to adhesion molecules and to transduce the signals generated from the receptors.

5. *Unanswered Questions*

One of the questions remained to be answered is why a 5' unbounded open reading frame exists in the cDNA sequence. One possibility would be that the cDNA does not represent the full length CD9 mRNA. Evidence that appears to support this concept is the detection by Northern Blotting of a 2.2 kb mRNA using the cDNA as a probe (see later chapter for detail). If this is the case, CD9 protein could be translated as a larger precursor protein and the precursor subsequently cleaved during post-translational modification. The cleavage would generate two protein products, the CD9 antigen and a second peptide. This second protein could either be functional (involved in CD9 related activity) or non-functional as in the case of pro-insulin. The lack of signal sequence by the CD9 peptide translated from the fifth start codon would also seem to

suggest that translation starts at an unidentified upstream ATG. Alternatively, it is also possible that the cDNA does represent the full length CD9 mRNA, and that the 5' ORF simply represents a less common event when the ORF is not translated due to lack of the neighboring DNA sequence to serve as a start codon. There are a few genes cloned that have a similar 5' unbounded ORF. With some, the non-coding nature of the ORF was confirmed by making antibody against the synthetic peptide according to the ORF sequence and testing whether the antibody reacted with any protein translated from the cDNA selected mRNAs (Loftus et al., 1987), or by obtaining the internal peptide sequence to determine the correct start site (Tyers et al., 1988).

Other questions regarding further characterization of the CD9 cDNA, and confirmation of its authenticity are presented in the next chapter.

CHAPTER VI

FURTHER CHARACTERIZATION OF CD9 cDNA

A. INTRODUCTION

Although the use of polyclonal antibody in cDNA cloning from λgt11 library has enabled, in most instances, the identification of the fusion proteins that would otherwise be impossible to identify with monoclonal antibody, problems may be encountered if the polyclonal antibody cross reacts with components other than the immunogen. These might include cross reactivity against the E. coli proteins and even β-galactosidase, as well as irrelevant proteins. False positive clones are frequently isolated as a result of these cross reactive antibodies and/or the protein non-specific epitopes. It is therefore extremely important to verify the authenticity of the clone which is identified with polyclonal antibody. One commonly used method to indirectly authenticate the clone involves producing the fusion protein from the recombinant λgt11 lysogen. The fusion protein is analyzed by electrophoresis to estimate the size of the foreign protein, which is then compared to that of the native protein. The fusion protein is also used to purify the polyclonal antibody originally used in library screening, and the purified antibody tested by Western blotting to determine whether it still recognizes the native protein. Alternatively, the cDNA is used to perform a Northern blot, and the level of mRNA detected by the cDNA compared to that of native protein expression. A more direct method to confirm the clone's authenticity involves subcloning the cDNA into an expression vector, and the immunobiochemical features of the protein product is then compared to that of the native protein. A variation of this method involves *in vitro* translation of the corresponding mRNA obtained either by selection of the cellular mRNAs with cDNA or by subcloning the cDNA into plasmid vector SP6. Alternatively, antibodies can be raised against the synthetic peptides that represent the predicted protein

hydrophilic regions, and the antibodies reacted with the native proteins. In addition, peptide cleavage sites within the predicted protein can be compared with those established by peptide mapping using the native antigen. The most direct approach to authenticate the cDNA however would be to obtain a partial amino acid sequence from either the amino-terminal of the native protein, or from the internal proteolytic fragment, and the amino acid sequence then compared to that predicted by the cDNA. Because of the concern over the cross-reactivity of the polyclonal antibody, most cDNAs isolated from λgt11 libraries have been directly or indirectly confirmed with one or several of the above mentioned methods.

Since CD9 cDNA was isolated from the λgt11 library with CD9 polyclonal antibody, efforts were made to confirm its authenticity using various approaches. In addition, the control of CD9 expression, and its genome structure were explored using the CD9 cDNA as a probe. Finally, the CD9 cDNA was used to isolate a second clone from the NALM-6 λgt11 cDNA library. The results from these experiments are presented in this chapter.

B. AUTHENTICATION OF THE CD9 cDNA CLONE

1. *Subcloning of the CD9 cDNA in Opposite Orientation into Expression Vector*

Large scale plasmid DNA preparation was carried out to isolate the 1.3 kb insert from the recombinant pUC19. The insert was excised out with Eco RI, separated by agarose gel electrophoresis, recovered by the "gene clean" method, and ligated to the expression vector pcEXV-3 which was cut with Eco RI as described previously. The construction and restriction map of the vector is presented in Figure 28. Colonies that grew on ampicillin plates were picked, and rapid plasmid DNA preparation was made from each colony, which was digested with Eco RI and analyzed by agarose gel electrophoresis for the presence of the 1.3 kb cDNA insert. As indicated in Figure 29,

both colonies examined had the expected insert, as compared with the wild-type vector which was also cleaved with Eco RI. DNAs from these two plasmids were purified on a large scale by the CsCl method, and the quality of the DNA analyzed by agarose gel electrophoresis. In addition, the orientation of each recombinant plasmid was determined by diagnostic restriction enzyme digestion followed by gel electrophoresis. Figure 30 is the restriction map of the 1.3 kb insert generated by computer program. While there is a single Nru I restriction site within the cDNA, there is no internal Hind III site. The DNA quality and the insert orientation analysis is presented in Figure 31a. Almost all the plasmid DNAs purified over the CsCl gradient were closed circular, as was evidenced by the single plasmid DNA band. Furthermore, since there is only one internal Nru I site in the cDNA but no Hind III site, while there is one Hind III site in the plasmid DNA upstream from the cloning site but no Nru I site, digestion with the two enzymes would generate a 1.0 kb fragment, or a 1.3 kb fragment from the recombinant plasmid DNA depending upon which orientation the insert had in relation to the Hind III site (Figure 31 b). The analysis indicated that the 1.3 kb cDNA inserts possessed by the two plasmids were in opposite orientations.

2. *Expression of the CD9 cDNA through Transfection*

Transfection of the 1.3 kb insert into NIH 3T3 cells which do not contain CD9 detectable with murine monoclonal antibodies to CD9 should enable the human gene product to be detected with the murine monoclonal. The closed circular expression vectors containing the 1.3 kb inserts in opposite orientations were introduced into NIH 3T3 cells by polybrene-assisted transfection, as described in materials and methods. After DMSO shocking and sodium butyrate treatment, the cells were harvested (3 days after the initial DNA transfection), lysed with extraction buffer, and analyzed by Western blotting with both monoclonal, and polyclonal antibody for the presence of CD9 antigen. Figure 32 shows the result of blotting. When monoclonal antibody was used

CD9 antigen was not detected in the transfected cells, but only in surface-positive NALM-6 cells and platelets. Since CD9 was not detected qualitatively it was possible that use of the polyclonal might reveal a quantitative increase in CD9. However, when polyclonal antibody was used, CD9 antigen was present in cells transfected with the cDNA in the proper orientation in amounts comparable to that of cells transfected with the cDNA in the opposite orientation.

Since CD9 could not be detected in transfected NIH 3T3 cells, the possibility of using the monkey kidney cell line COS-7 cells as an alternative DNA transfection recipient was explored since COS-7 cells are known to lack surface CD9 expression detectable by monoclonal antibody. The COS-7 cells were intrinsically labeled with ^{3}H-leucine, lysed with extraction buffer, and the presence of CD9 determined by immunoprecipitation with polyclonal antibody. Figure 33 is the result of the immunoprecipitation. Unfortunately, the COS-7 cells also contained CD9 detectable by the polyclonal antibody limiting their utility for studying CD9 expression.

3. *Further Purification of CD9 for Microsequencing*

CD9 antigen was purified from platelet lysate by the mAb 50H.19 affinity chromatography as described in Chapter II. The antigen was further purified away from the coprecipitating proteins by SDS-PAGE followed by electroelution. The quality and the concentration of the purified antigen was then analyzed by SDS-PAGE followed by silver staining. Figure 34 is the result of the SDS-PAGE and silver staining. Compared to the column eluate, no coprecipitates were detected. Interestingly, a protein with a molecular weight of 45,000 was barely seen in the lane loaded with the electroeluted CD9 protein. The amount of protein in each lane was determined by comparison with the BSA protein marker (not shown). 1.3 mg of the electroeluted CD9 was obtained from 100 bags of the out-dated platelets.

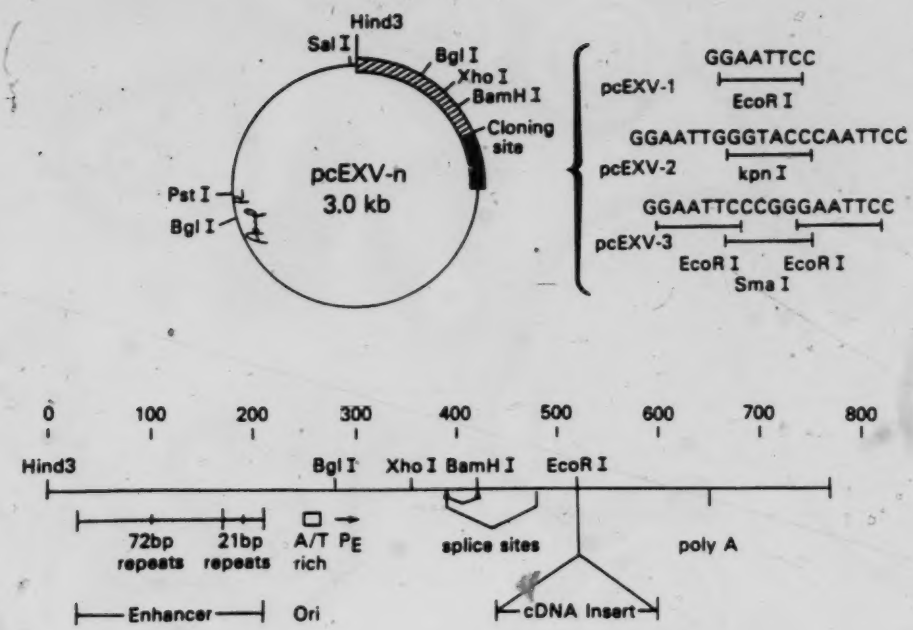


Figure 28. Features and restriction endonuclease map of the cDNA expression vector pcEXV-3. The vector has a unique Eco RI site at the cloning site.The linear map at the bottom of the figure is a description of the SVC40 sequences that are present in the vector, which contain the SV40 enhancer, replication origin (Ori), early promotor (P_E), and splice signals 5' to the cDNA insert as well as a polyadenylation signal [polyA] downstream. The remaining sequences in the vector are derived from pBR322 and contain the ampicillin resistance gene (amp).

For detailed description of the vector, see Miller and Germain (1986).

Figure 29. Subcloning of the 1.3 kb CD9 cDNA into expression vector pcEXV-3. The 1.3 kb CD9 cDNA was excised from the pUC19 with Eco RI, and ligated to the expression vector pcEXV-3 previously cut with Eco RI and dephosphorylated. The religated plasmids were plated out on LB plates containing 50 μg/ml ampicillin. DNAs were isolated from the colonies that grew on ampicillin plates, digested with Eco RI, and analyzed by 0.8% agarose gel electrophoresis followed by staining with ethidium bromide. Lame M was loaded with the DNA size markers λ DNA cleaved with Hind III. Lane a was loaded with the wild-type pcEXV-3 cut with Eco RI, while Lane b and c contained recombinant pcEXV-3 cut with Eco RI. Both recombinant plasmid carried the 1.3 kb cDNA insert.

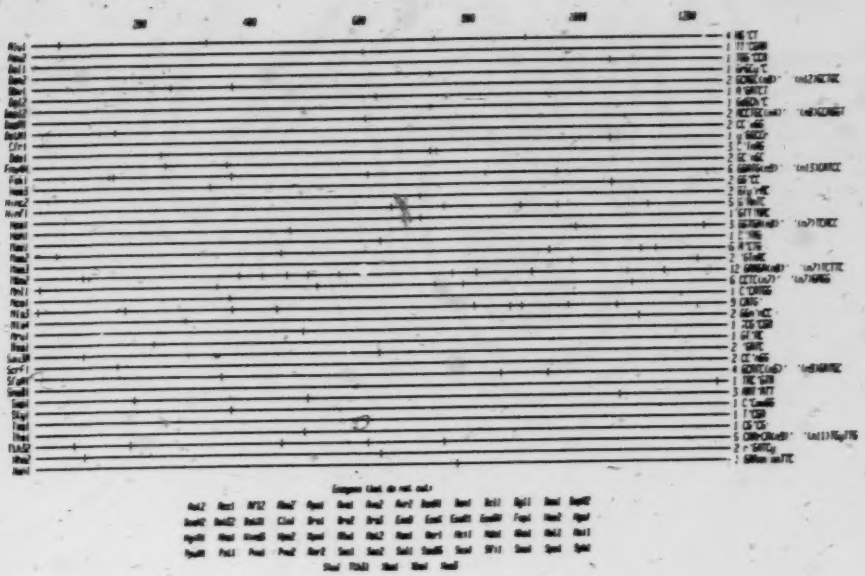
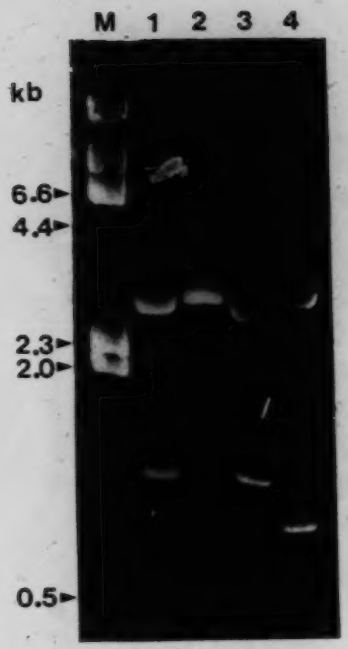


Figure 30. Restriction map of the 1.3 kb CD9 cDNA insert. The restriction map of the 1.3 kb CD9 cDNA was generated by a computer program. The distance of the nucleotide from the 5' end is indicated by the scale on top of the map. The restriction enzymes that cleave within the cDNA are listed on both sides of the cDNA. The frequency of the each restriction site is presented as the vertical bars. The enzymes that do not cleave the cDNA are listed on the bottom of the map. While there are two Hae 3 sites and one Nru I site within the cDNA, no Hind III site exists within the sequence.

a.



b.

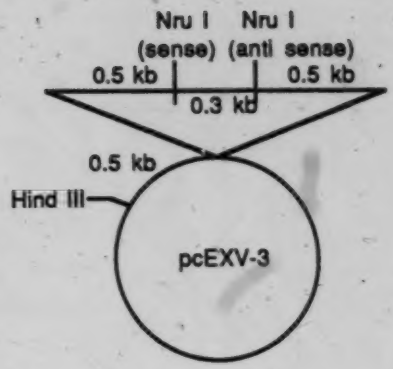


Figure 31. Analysis of the insert orientation by restriction enzyme digestion. Figure 31b is a simplified restriction map of the recombinant pcEXV-3 carrying the 1.3 kb CD9 cDNA. Digestion with Hind III (H) plus Nru I (N) would generate a 1.0 kb fragment and a 3.3 kb fragment, if the insert is in sense orientation. On the other hand, fragments with sizes of 1.3 kb and 3.0 kb would be generated if the insert is in anti-sense orientation. Figure 31a shows the result of diagnostic restriction enzyme digestion. Lane M was loaded with the DNA size markers λDNA cut with Hind III. Lane 1 was loaded with the Eco RI digested plasmid in sense orientation, while Lane 2 was loaded with the uncut pcEXV-3 vector carrying the 1.3 kb cDNA in sense orientation purified over a CsCl gradient as a control. Lane 3 and 4 were loaded with the recombinant pcEXV-3 cut with Hind III and Nru I. As expected, a 1.3 kb DNA fragment was excised from the vector.The plasmid in Lane 4 gave rise to 1.0 kb and 3.3 kb fragments upon enzyme digestion, hence was in the sense orientation, while the one in Lane 3 gave rise to 1.3 kb and 3.0 kb fragments, hence was in anti-sense orientation. Almost all the plasmids were close circular DNAs.

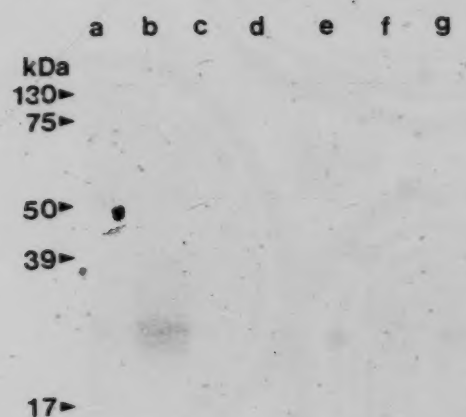


Figure 32. Analysis of the NIH 3T3 cells transfected with CD9 cDNA by Western blotting. The recombinant pcEXV-3 carrying the CD9 cDNA was introduced into the NIH 3T3 cells by the polybrene-assisted transfection method. Cell lysates were prepared, reduced, and denatured by boiling in the presence of SDS and 2-ME. The proteins were separated by SDS-PAGE on a 12.5% acrylamide gel, blotted onto a nitrocellulose filter, and reacted with the 1/400 dilution of CD9 polyclonal antibody. Although the color of the filter was faded by the time the photo was taken, it can still be used to illustrate the result of the transfection. Lane a to g were loaded with lysates from NALM-6 cells, platelets, HepG2 cells, Jurkat cells, NIH 3T3 cells, NIH 3T3 cells (transfected with the CD9 cDNA in anti-sense orientation), and NIH 3T3 cells (transfected with the CD9 cDNA in sense orientation). CD9 antigen was detected in surface positive cells NALM-6, platelets, and HepG2. Although CD9 was detected in the NIH 3T3 cells transfected with the cDNA in sense orientation (Lane g), it was also present in the NIH 3T3 cells transfected with the cDNA in anti-sense orientation (Lane f). Furthermore, CD9 was detected in the surface CD9 negative cell lines, NIH 3T3 cells (non-transfected) (Lane e) and Jurkat cells (Lane d).

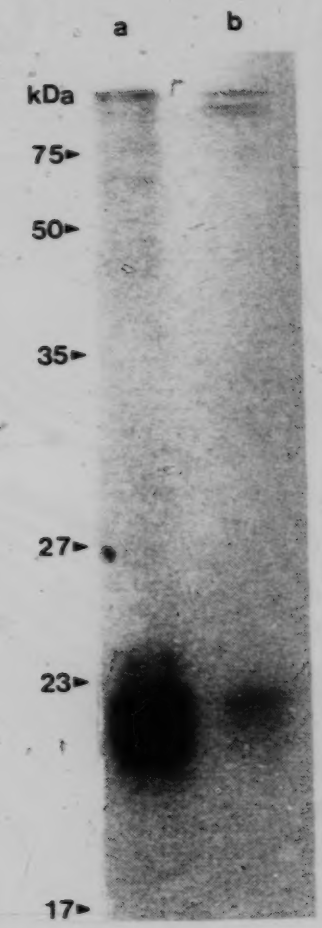


Figure 33. Immunoprecipitation of COS-7 cell lysate with CD9 polyclonal antibody. NALM-6 cells and COS-7 cells were intrinsically labelled with ^{3}H-leucine. Lysates were prepared from radiolabelled cells, and then incubated with CD9 polyclonal antibody couple to protein A-sepharose CL-4B. The bound proteins were eluted from the beads by boiling in sample buffer, separated by SDS-PAGE on a 12.5% acrylamide gel, and visualized by fluorography. Lane a and Lane b were loaded with the precipitates from NALM-6 and from COS-7 cells respectively. CD9 antigen (both the major band of gp22 and the minor band of gp24) was seen in precipitate from NALM-6. A major band of gp22 was also clearly seen in precipitate from COS-7 cells (a surface CD9 negative cell line).

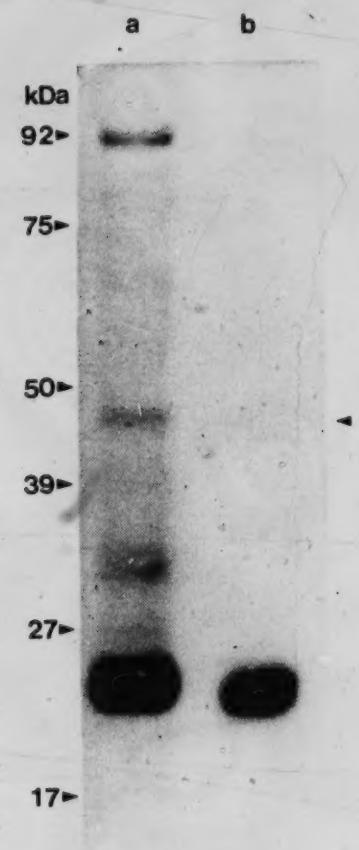


Figure 34. Analysis of the electroeluted CD9 by SDS-PAGE and silver staining. Column eluate from platelets separated by mAb 50H.19 affinity chromatography was further purified by SDS-PAGE on a preparative 12.5% acrylamide gel, the gel stained with Coomassie blue, and the band containing the gp22 excised. CD9 was recovered from the gel by electroelution. A fraction of the highly purified protein was reduced and denatured by boiling in the presence of SDS and 2-ME, and then analyzed by SDS-PAGE followed by silver staining. Lane a was loaded with the column eluate from platelets before the electroelution, while Lane b was loaded with the highly purified CD9 protein. No other coprecipitates were detected in the electroeluted protein, except a minor protein with a molecular weight of 45 kDa (arrow).

4. *Generation of Antibodies against the Synthetic Peptides*

Amino acid sequence of the CD9 protein was deduced from the CD9 cDNA. Two synthetic peptides, EDLTRLKET and QQESYTYKDP, were synthesized by the Alberta Peptide Institute. The first peptide represents a hydrophilic region at the amino-terminal of the CD9 protein, right after a V8 cleavage site. The second peptide represents a hydrophilic region in the middle of the protein, immediately after a formic acid cleavage site. The synthesized peptides were purified by reverse-phase HPLC, and the purity and the peptide sequence were verified by amino acid analysis. The KLH-conjugated peptides were then used to immunize rabbits in order to obtain antibodies against the synthetic peptides. Preliminary antisera characterization by ELISA indicates that one of the rabbit developed high titre antibody against the synthetic peptide. Furthermore, the rabbit antisera seems to be specific for native CD9 antigen, since 82% CD9-positive NALM-6 cells were stained by the antisera (plus the fluorescein-conjugated second antibody) while only 12% surface CD9 negative Raji cells were stained, when analyzed by FACS.

C. CD9 GENOME STRUCTURE AS REVEALED BY SOUTHERN BLOTTING

Genomic DNAs were isolated from human thymus and placenta tissues, and from Jurkat cells. The subsequent Southern blot analysis was performed by Dr. Jennifer Shaw. The DNAs were digested to completion with either Hind III or Pst I, and the digested DNAs analyzed with the ^{32}P-labeled 1.3 kb cDNA insert and its 5' and 3' end fragments by Southern blotting. The 350 bp 5' and 200 bp 3' end fragments were generated by digesting the 1.3 kb cDNA with restriction endonuclease Hae3 to completion, separating the DNA fragments through agarose gel electrophoresis, and recovering the desired DNAs from the excised gel bands. As presented in Figure 35, multiple bands were recognized by the full length cDNA probe whether the genomic

DNA was cut with Hind III or Eco RI. However at least three bands were still recognized by the 3' end probe, and five bands recognized by the 5' end probe in DNA cut with Hind III, and at least two bands were recognized by the two probes in DNA cut with Pst I. Furthermore when genomic DNAs was digested with Hind III and probed with either the full cDNA or the 5' end probe, thymus DNA seemed to lack the 8.0 kb band found in DNAs from other tissues, while Jurkat DNA gave an extra 4.0 kb band. Initially, it was thought that CD9 gene may have tissue specific restriction fragment length polymorphism. Repeated experiments using thymus DNAs from different individuals revealed that the presence or absence of that particular fragment was individual specific rather tissue specific. As for the the extra 4.0 kb band found in Jurkat cells, further experiment is needed to determine if the extra Hind III site is due to mutation in Jurkat cells or is specific to normal T-cells from certain or all individuals.

D. CD9 EXPRESSION AS REVEALED BY NORTHERN BLOTTING

Total cellular RNAs were isolated from surface CD9 positive NALM-6, SKOSC, JEG, and SW480 cells, as well as from the surface negative Raji and Wacko cells by guanidine thiocyanate-CsCl method. Poly A^+ mRNAs were obtained from NALM-6 and JEG total cellular RNAs by subsequent oligo-dT chromatography. These RNAs were fractionated by agarose gel (10 μg per lane) and analyzed by a Northern blotting using the ^{32}P-labeled 1.3 kb cDNA as a probe. Figure 36 is the result of the Northern blotting. The cDNA identified a mRNA with a size of 2.2 kb, a size similar to that of 18S ribosomal RNA. At least 5 fold stronger signals were obtained from NALM-6 and JEG poly A^+ mRNAs as compared to their total cellular RNAs, indicating that the signal was not derived from the cDNA that hybridized to the 18S ribosomal RNA. Surprisingly the CD9 messengers were also detected in the surface CD9 negative Wacko and Raji cells.

Since CD9 antigen is known to be induced in the activated T-cells, the level of the CD9 mRNAs in resting and activated T-cells was studied by Northern blotting, using the ^{32}P-labeled 1.3 kb insert as a probe. Figure 37 is the result of the Northern blotting. At least three-fold increase in the CD9 mRNA occurred in the T-cells stimulated with 5 μg/ml of PMA and 3 μg/ml of PHA, while at least five-fold increase occurred in the T-cells stimulated with 5 μg/ml of PMA and 10 μg/ml of PHA, as compared to the unstimulated control. The experiment not only confirmed the earlier observation of CD9 induction in activated T-cells at the protein level, it also revealed that the induction occurred in a dose-dependent manner. A similar increase in CD9 messengers in the stimulated T-cells was observed in cytodot hybridization using the ^{32}P-labeled 1.3 kb cDNA as probe.

The wide presence of CD9 antigen in tumor cells also prompted an experiment designed to study CD9's expression in transformed cells. Total cellular RNAs were isolated from the NIH 3T3 cells, from the 3T3 cells stably transfected with a plasmid cDNA expression vector pSV2neo, and from the 3T3 cells stably transfected with the pSV2neo carrying the cellular ras^{Ha} oncogene (c-ras^{Ha} onc, whose transcription is driven by the SV40 early promoter carried by the plasmid). The RNAs were analyzed by Northern blotting, using the ^{32}P-labeled 1.3 kb CD9 cDNA as probe (Figure 38). While transfection by the pSV2neo vector did not produce an increase in CD9 mRNA transfection of the pSV2neo-ras onc resulted in a clear increase in the level of the messenger. In contrast, control hybridization of the same filter (after boiling in water for 10 minutes to remove previously bound DNA probes) with the ^{32}P-labeled human actin probe indicated that the increase in mRNA is specific for CD9, since actin mRNA appeared to be slightly decreased as a result of transfection with both the pSVneo vector and the pSVneo vector carrying the c-ras^{Ha} onc.

E. ISOLATION OF A SECOND CD9 cDNA CLONE FROM A NALM-6 LIBRARY

1. *Identification of a 0.7 kb cDNA Clone with the 1.3 kb cDNA Probe*

2×10^6 p.f.u. phage from NALM-6 λgt11 library were plated out on 20 large LB plates (pH 7.5), and duplicate nitrocellulose filters lifted from the phage plaques were screened with the ^{32}P-labeled 1.3 kb cDNA probe, as described in Chapter II. Five positive plaques were initially identified in the first round screening, of which only one clone remained positive in the following rounds of screening, as presented in Figure 39. Rapid phage DNA preparation was made from this positive clone, the DNA digested with Eco RI to completion, and analyzed by agarose gel electrophoresis for the presence of any cDNA insert. Figure 40 is the result of the analysis. A cDNA with a length of 0.7 kb was clearly seen.

2. *The 0.7 kb cDNA Insert too could not be Excised out with Eco RI*

Large scale phage DNA preparation was carried out to isolate the cDNA insert for subcloning and sequencing. The 0.7 kb insert however could no longer be cut out with Eco RI. A diagnostic digestion with restriction enzyme Kpn I and/or Sst I was again performed to determine the presence of the cDNA insert. The digested DNAs were then analyzed by agarose gel electrophoresis. Figure 41 is the result of the insert analysis. Besides the phage arms, 4 major DNA bands are clearly seen (1.5 kb, 2.8 kb, 4.5 kb, and 8.0 kb), confirming that the cDNA insert was inside the phage DNA. Interestingly, a minor 2.1 kb DNA band was also seen, suggesting that the recombinant phage DNA was contaminated with that of the wild-type, although homogeneous recombinant phage (from after the third round screening) were used for large scale DNA isolation.

The 2.8 kb fragment was excised out from the phage DNA, and subcloned into the pUC19. Recombinant pUC19 carrying the 2.8 kb fragment was identified by restriction mapping of the DNAs isolated from the white colonies. However, unlike the problem encountered previously with the first CD9 cDNA clone, the 0.7 kb cDNA insert could

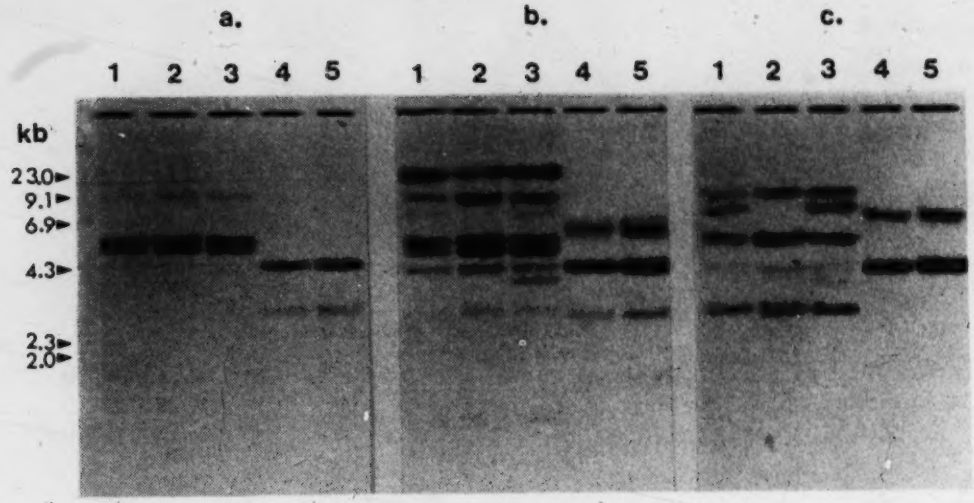


Figure 35. Analysis of the CD9 genome structure by Southern blotting. Genomic DNAs were isolated from human placenta and thymus tissues, and from Jurkat cells. The DNAs were digested to completion with Hind III or Pst I, separated by electrophoresis on a 0.8% agarose gel, blotted onto a nitrocellulose filter, and hybridized to ^{32}P-labelled 1.3 kb CD9 cDNA (Figure 35 b), or a 5' end 350 bp fragment (Figure 35 c), or a 3' end 200 bp fragment (Figure 35 a). Both the 5' and the 3' end fragments were generated from the 1.3 kb cDNA by digestion with Hae 3. Lane 1, 2, and 3 in each gel were loaded with the placenta, thymus, and Jurkat DNA respectively, all of which were cleaved with Hind III. Lane 4 and 5 in each gel were loaded with placenta and thymus DNA respectively, both were cut with Pst I. At least 7 to 8 bands were seen in Hind III digested DNA when probed with full cDNA probe, 4 to 5 bands were seen when the same DNAs were probed with the 5' end 350 bp fragment, while 3 bands were seen when the 3' end 200 bp fragment probe was used. As for the Pst I digested DNAs, at least 3 bands were detected when probed with the full cDNA, while two bands were detected when the 5' or 3' end probes were used. Thymus DNA did not give rise to an 8 kb band when cleaved with Hind III and probed with either the full length cDNA or the 5' end probe. Jurkat DNA on the other hand give rise to an extra 4.0 kb band under similar conditions.

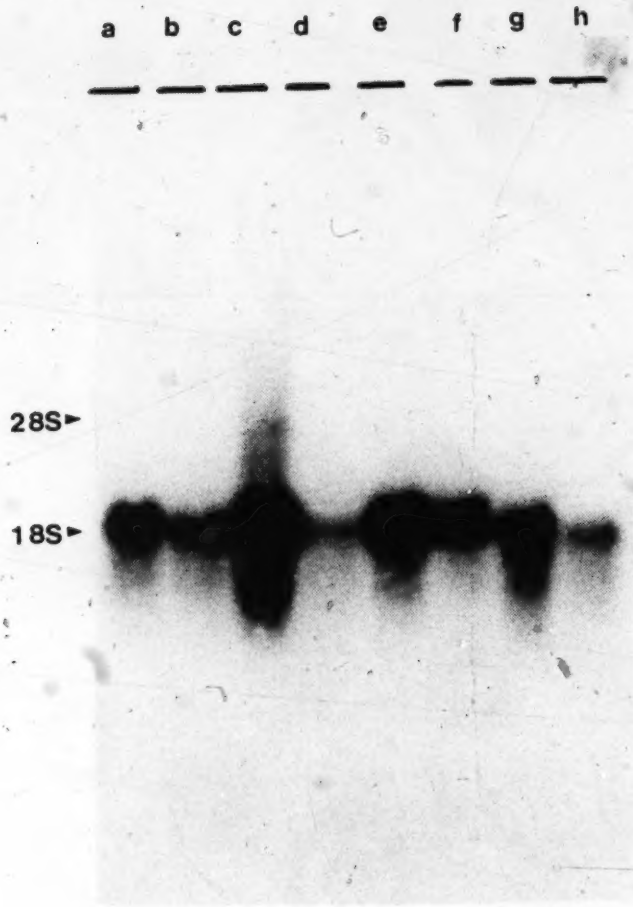


Figure 36. Analysis of CD9 expression by Northern blotting. Total cellular and poly A^+ mRNAs were isolated from both surface CD9 positive and surface CD9 negative cells. The RNAs were separated by electrophoresis on a denaturing 0.8% agarose gel, blotted onto a nitrocellulose filter, and hybridized to the ^{32}P-labelled 1.3 kb CD9 cDNA probe. The probe hybridized to a 2.2 kb mRNA in the lanes loaded with RNAs from surface CD9 positive cells, NALM-6 (Lane b), JEG (Lane f), SKOSC (Lane g), and SW480 (Lane h). Poly A^+ mRNA from NALM-6 (Lane c) and JEG (Lane e) gave rise to much stronger signals compared to the total RNAs, indicating that the hybridization was therefore not due to non-specific binding to the 18S rRNA. In addition, the probe also hybridized to a 2.2 kb mRNA from cells lacking surface CD9 antigen, such as Wacko (Lane a) and Raji (Lane d).

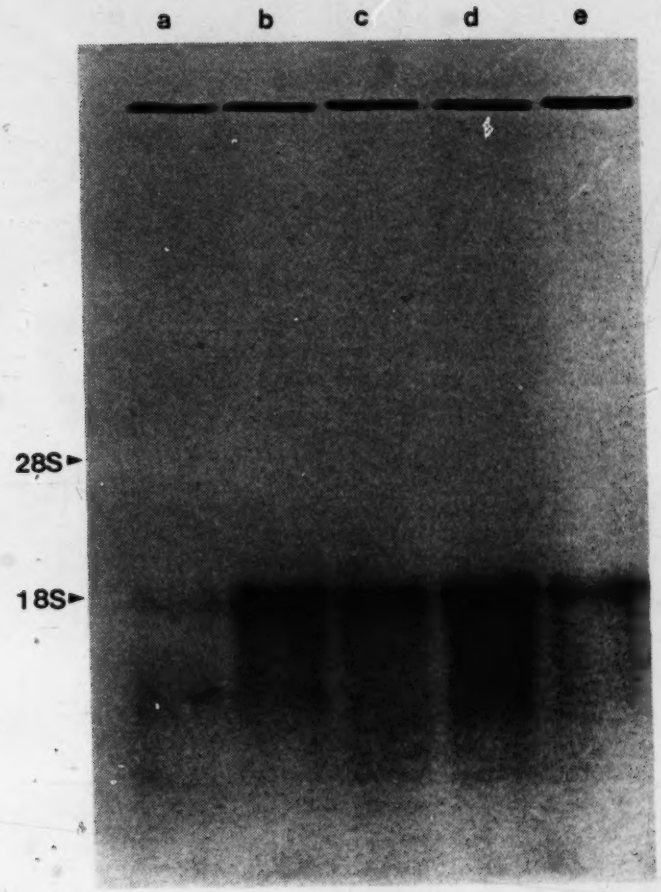


Figure 37. Induction of CD9 mRNA upon T-cell activation. Total cellular RNAs were isolated from the purified non-stimulated T-cells, and from the T-cells stimulated with PHA + PMA. The RNAs were analyzed by Northern blotting performed as in Figure 35, using the 1.3 kb CD9 cDNA as probe. Lane a was loaded with the total RNA from resting T cells, wile Lane b and c were loaded with the RNAs from T cells stimulated with 5 μg/ml PMA and 10 μg/ml PHA and with 5 μg/ml PMA and 3 μg/ml PHA respectively. Lane d and e were loaded with the RNAs from activated T cells similarly produced in a separate experiment. At least a three-fold increase in CD9 mRNA was found in the T-cells stimulated with 5 μg/ml PMA and 3 μg/ml PHA (Lane c and e), and a five-fold increase in T-cells stimulated with 5 μg/ml PMA and 10 μg/ml PHA (Lane b and d), compared to freshly prepared unstimulated T-cell controls (Lane a).

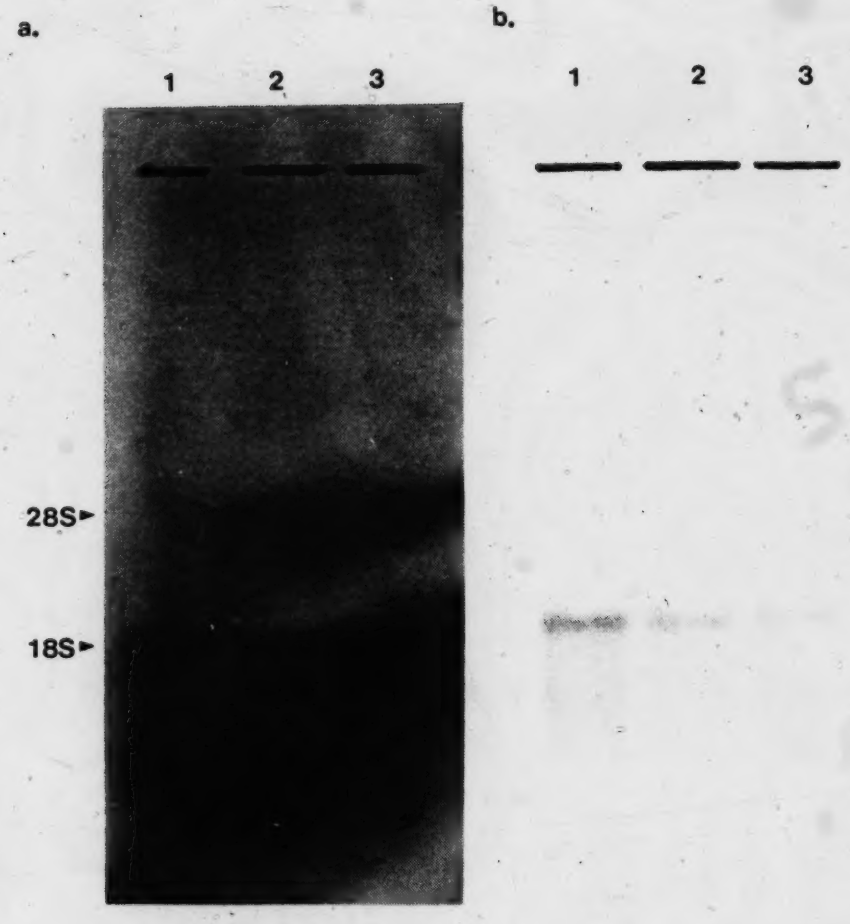


Figure 38. Induction of CD9 mRNA upon transformation of 3T3 cells by an activated c-ras^{Ha} oncogene. Total cellular RNAs were isolated from NIH 3T3 cells, NIH 3T3 cells transfected with pSV2neo vector, and NIH 3T3 cells transfected with pSV2neo-ras. The RNAs were analyzed by Northern blotting performed similarly to Figure 36, using the 1.3 kb CD9 cDNA as probe (Figure 37a). While there was no difference in CD9 mRNA expression between the pSV2neo transfected (Lane 1) and non-transfected 3T3 cells (Lane 2), at least three-fold increase in CD9 messengers was detected in the pSV2neo-ras transfected 3T3 cells (Lane 3). The same filter was also hybridized to a human actin probe (Figure 38b). Compared to the non-transfected 3T3 cells (Lane 1), a slight decrease in actin mRNA accompanied transfection with pSVneo (Lane 2) and with pSVneo carrying the c-ras^{Ha} oncogene (Lane 3).

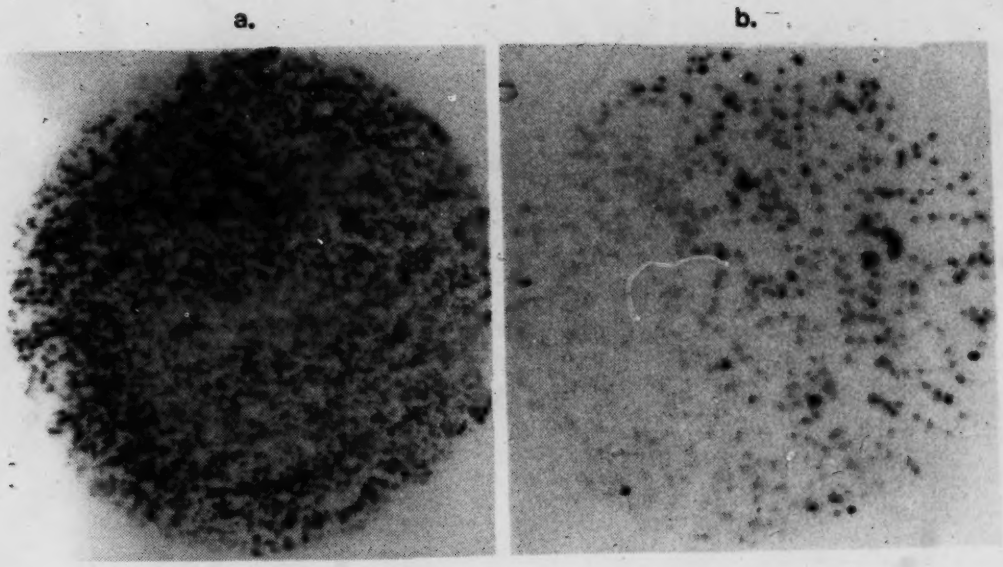


Figure 39. Isolation of a second CD9 cDNA clone from a NALM-6 λgt11 library with our cDNA probe. 2 x 10^6 p.f.u. phages from the NALM-6 library were plated out on large LB plates and blotted onto IPTG-free nitrocellulose filters. The phage DNAs were denatured, neutralized, fixed, and then screened with the ^{32}P-labelled 1.3 kb CD9 cDNA, which resulted in the identification of a second CD9 cDNA clone (Figure 39a). The positive plaque plus the surrounding plaques were picked, and used to perform a second round screening with the same probe (Figure 39b). A well-isolated positive plaque was finally selected to carry out a third round screening to ensure that a homogeneous population of positive clones was obtained.

Figure 40. Analysis of the second CD9 cDNA clone by agarose gel electrophoresis. DNA was isolated from the recombinant phage, digested with Eco RI to completion, and analyzed by 0.8% agarose gel electrophoresis followed by staining with ethidium bromide. Lane M was loaded with the DNA size markers λDNA cut with Hind III. Lane a was loaded with the untreated recombinant phage DNA, while lane b was loaded with the same DNA digested with Eco RI. A 0.7 kb insert was clearly seen in Lane b (arrow).

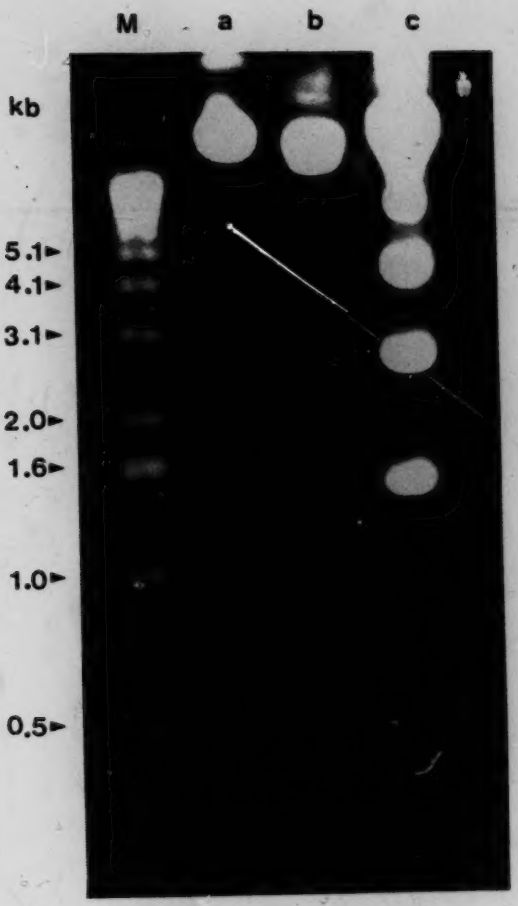


Figure 41. Analysis of the recombinant phage carrying the 0.7 kb CD9 cDNA insert by diagnostic restriction enzyme digestion. DNA isolated from the CD9 positive phage clone was digested with Eco RI, or Kpn I, or Kpn I plus Sst I. The digested DNAs were analyzed by electrophoresis with a 0.8% agarose gel followed by staining with ethidium bromide. Lane M was loaded with the BRL kilo-base DNA size markers. Lane a to c were loaded with the recombinant phage DNA cut with Eco RI, Kpn I, and Kpn I plus Sst I respectively. Excluding the phage arms, or intact phage DNA, digestion with Eco RI did not generate any visible cDNA insert (Lane a), digestion with Kpn I generated one DNA fragment (1.5 kb) (Lane b), while digestion with Kpn I plus Sst I generated four DNA fragments (1.5 kb, 2.8 kb, 4.5 kb, and 8.0 kb respectively) (Lane c). Interestingly, a minor band of 2.1 kb fragment was also seen in the DNA cleaved with Kpn I plus Sst I (Lane c).

Please refer to Figure 15 b for the restriction map of the wild-type λgt11 DNA.

still not be recovered form the 2.8 kb fragment, even though the latter was subcloned into the plasmid vector (result not shown).

3. *Sequencing of the Second CD9 cDNA*

Large amounts of the 2.8 kb fragment will be obtained through preparative agarose gel electrophoresis using the Kpn I and Sst I digested recombinant plasmid DNA. The linear double stranded DNAs will be denatured, and used directly as sequencing templates to perform the dideoxy chain termination sequencing using the λgt11 primers.

Alternatively, the 2.8 kb fragment is also being subcloned directly into the M13mp18 and M13mp19 vectors that are previously digested with Kpn I and Sst I. Single stranded DNA would then be prepared and used to perform the dideoxy chain termination sequencing.

F. Discussion

Of the various methods used to confirm the identity of the positive clone isolated from λgt11 cDNA libraries with polyclonal antibodies, matching the predicted amino acid sequence with that established from microsequencing the amino-terminus (N-terminus) of the native protein is probably the most direct approach. However, due to modification of the amino acids at the N-terminus, not all proteins can be sequenced from the amino-terminus (as is the case with CD9 antigen). Of the repeated microsequencings from the N-terminus of the CD9 protein, only one resulted in a 7 amino acid sequence which represented a minor protein component. This minor component is probably a non-human protein, since the ^{32}P-labeled oligonucleotides synthesized based on the amino acid sequence failed to bind human genomic DNA by Southern blotting. Therefore it will be necessary to generate peptides from intact CD9 antigen in order to obtain partial amino acid sequence for the purposes of clone confirmation.

While amino acid sequence information was being sought to confirm the identity of the isolated cDNA, other clone authentication methods were attempted. Efforts were made to produce the fusion protein from the recombinant phage carrying the CD9 cDNA. However, the lysogeny could not be established because no lytic growth could be observed with E. coli Y1089 when the bacteria were grown at 42°C, while the same phage stock produced high density plaques in E. coli Y1090 control similarly transformed and grown. Replacing the plating bacteria with the ones from different sources did not overcome the problem. Although the fusion protein approach would confirm that the protein identified bears the specific epitope, it could not exclude the possibility of identifying irrelevant proteins carrying the same epitope. Since rescreening of the CD9 positive clone with antibodies pre-adsorbed with cells differing in CD9 antigen expression had already provided the information which the fusion protein approach would answer, emphasis was placed on other clone confirmation methods instead.

The result of transient CD9 expression in NIH 3T3 cell is both disappointing and interesting. NIH 3T3 cell was chosen as recipient cell for CD9 expression experiment because it was known to be negative for CD9 antigen, as indicated by studies with monoclonal antibodies. However, when cell lysate was prepared from these CD9 negative cells, including the Jurkat cells, and analyzed by Western blotting using polyclonal antibody raised against the denatured protein, CD9 was unmistakably found. This discrepancy could only be explained by the difference in recognition pattern of the two antibodies. The epitope defined by CD9 monoclonal antibodies 50H.19, ALB-6, and BA2 contains a disulfide bridge, which is critical for recognition by these monoclonal antibodies. Therefore, while polyclonal antibody is able to recognize the denatured CD9 protein, monoclonal antibody only recognizes the properly folded molecule. The finding of the CD9 protein in CD9 negative cells defined by monoclonal

antibody indicates that CD9 proteins do exist in these cells. Furthermore, the fact that the polyclonal antibody failed to reveal these proteins on the cell surface, as was evidenced by the negative immunofluorescence staining, further suggests that the protein is located subcellularly. In order to be recognized by the monoclonal antibody, the internal protein may have to be transported to the plasma membrane where it achieves its proper folding. It appears therefore, that the expression of the surface CD9 antigen is controlled at a post-translational level as well. The existence of CD9 mRNA in surface CD9 negative cells supports this concept. Because of the "background" CD9 protein, it is difficult to interpret the result of the transfection experiment using Western blotting. Since CD9 expression in transfected cells is affected not only by the efficiency of the transfection but also by the efficiency of transcription and translation, we are unlikely to observe an obvious difference between the two populations in terms of the CD9 expression, unless most transfected cells highly express the exogenous CD9 cDNAs.

The multiple DNA bands visualized on hybridization of the cDNA probe on a Southern blot to DNA digested with Hind III indicates that genomic CD9 DNA comprises about 60 kb. This would be compatible with the existence of multiple exons, and with the possibility of multiple gene copies, or the presence of multiple genes. Reprobing with the 5' end cDNA probe, and the 3' end cDNA probe enabled the detection of two fragments recognized by both probes indicating that at least two genes, or gene copies might be present.

The significance of the restriction fragment length polymorphism exhibited by CD9 cDNA is interesting, but difficult to evaluate unless further studies are carried out to see whether loss of particular Hind III sites correlate with any genetic disorder, especially disorders in cell adhesion or activation functions.

The finding of CD9 mRNA in cells lacking surface CD9 antigen complements the finding from the Western blotting using polyclonal antibody, suggesting that CD9

expression may be controlled at both the transcriptional and post-transcriptional levels. The seemingly ubiquitous presence of CD9 mRNA is probably reflected by the fundamental physiological role played by CD9 protein. To determine the rate of transcription in each cell type, it would be necessary to conduct an RNA "run-off" assay using the genomic CD9 DNAs. It is clear from the T-cell activation and from the *ras* oncogene transfection experiments that the transcription of CD9 mRNA can be up-regulated upon cell activation, which may explain the expression of the surface CD9 in activated T-cells. However, the presence of the surface CD9 may not be explained by the increase in mRNA alone, since the surface antigen negative T-cells also possess CD9 messengers. Furthermore, even in cells with increased CD9 messengers induced by transfection with the *ras* oncogene the surface CD9 antigen is still absent. It seems likely that other proteins produced as a result of cell activation may be required for the surface expression of the CD9 proteins..

Finally, the purpose of screening the NALM-6 λgt11 library is many fold. Although mRNAs from different cell types identified by the CD9 cDNA were found to have a similar size, the tissue specific glycosylation of CD9 protein does suggest the possible existence of diversified CD9 protein sequences. Cloning and sequencing the CD9 cDNA from NALM-6 cells would help to clarify whether the different CD9 components gp 22, gp 24, and gp27 are products of different but closely related CD9 genes or of the single CD9 gene. Furthermore, since the first CD9 cDNA isolated has an unbound 5' end, screening with the cDNA probe may result in the isolation of a longer cDNA which may give more sequence information on the 5' end. Screening with the cDNA probe may result in the isolation of CD9 related sequences as well.

The problem of excising out the cDNA with Eco RI from the phage DNA was not new. However, the inability to cut out the insert even after the 2.8 kb fragment was subcloned into the pUC19 vector was puzzling. Since there was no other phage DNA

present, and the fragment was now subcloned into plasmid vector, neither Eco RI site blockade nor Eco RI site methylation could explain the inability to cut out the insert with Eco RI. One remaining possibility would be that the sites were modified during library construction, although it is very unlikely that both Eco RI sites could be similarly modified. A better answer will be provided once the insert is sequenced, since the presence of the intact or modified Eco RI sites on both ends could then be verified.

Thanks to the availability of the λgt11 sequencing primers, the insert may be sequenced directly using the double-stranded DNA as templates. Alternatively, the 2.8 kb fragment could be subcloned into the M13 vector, and single-stranded DNA from the recombinant M13 carrying the 2.8 kb insert used as template for sequencing. A possible concern with the second approach arises from the fact that the cDNA was originally cloned into the lac-Z gene of the λgt11 and the cDNA plus the flanking lac-Z DNA was again subcloned into the lac-Z gene of M13. Two priming sites, one on each lac-Z DNA, may prime the sequencing reaction, making the result impossible to interpret. However, a literature search aimed at finding out the difference between the two lac-Z regions used to insert the cDNA confirmed that this approach was indeed practical. The cloning site in λgt11 is constructed in the far 3' end of the intact lac-Z gene, while this region is totally missing in M13 because a truncated lac-Z gene containing only a short stretch of 5' end DNA is built into the vector instead.

CHAPTER VII

SUMMARY AND CONCLUSIONS

CD9 antigen is distinguished from all other described cell surface nonintegral glycoproteins by its novel attachment solely by ester-linked long chain fatty acids, and by the presence of multiple reversible acylation sites. Furthermore, the role played by CD9 in cell activation and in cell-cell or cell-environment interaction, as suggested by its tissue distribution pattern and confirmed by studies with CD9 antibodies, has established the protein as a molecule with significant biological importance. Although the immunobiochemical features of the protein had been extensively studied over the past few years, a better understanding of its structure and function still awaits the characterization of the gene encoding the protein. This study was designed to address the issue of molecular cloning of the CD9 cDNA.

One of the most common methods utilized to clone cDNAs representing low abundance mRNA from a cDNA library involves screening the library with ^{32}P-labeled oligonucleotide probes chemically synthesized according to the partial amino acid sequence. Alternatively, λgt11 could be used as the cloning vector for constructing the expression cDNA library so that the identification of the CD9 positive clone could be based on the protein product instead. The strategy used in this study was to purify a large amount of CD9 protein, and to construct a λgt11 cDNA library using mRNAs isolated from NALM-6 cells which are high producers of CD9 antigen. To identify the CD9 positive clone, mAb 50H.19 would be used to initially screen the NALM-6 λgt11 library, which, if it failed, would be followed by screening with polyclonal antibody raised against the denatured CD9 antigen. The purified antigen would also be used to generate a partial amino acid sequence from either the amino-terminus or the internal peptide fragment of the native CD9 protein. The amino acid sequence would be valuable

in confirming the authenticity of the CD9 clone identified by antibodies. Furthermore, as a back-up measure, oligonucleotides could be synthesized based on the amino acid sequence, and then be used to identify the CD9 positive clone.

One of the biggest hurdles to overcome in the study was to find a way to purify sufficient CD9 protein. Tremendous difficulty was encountered during the initial purification because of the extreme hydrophobic nature of the antigen, which may be explained by its high content of fatty acid ligands. The protein bound non-specifically to the laboratory-ware and therefore made recovery very difficult. The problem was compounded by the need to conduct time-consuming large-scale tissue culture to provide a sufficient supply of CD9 antigen bearing cells. Although removal of the deoxycholate from the elution buffer made concentrating the column-eluate possible, a process necessary for visualizing the purified antigen from NALM-6 cells, a turning point came when it was discovered that CD9 was highly expressed on human platelets, which were readily available from the Red Cross Blood Transfusion Centre. Furthermore, it was found that coating the laboratory-ware with CD9-containing cell lysate would greatly reduce the non-specific binding of the CD9 antigen. The almost unlimited supply of CD9 antigen source, combined with a much more efficient recovery from the mAb affinity chromatography finally made the purification of large amounts of CD9 antigen a reality.

The problem experienced in the initial antigen purification was the cause for early failure in rabbit immunization. The availability of large amounts CD9 antigen from human platelets enabled the visualization of the protein by silver staining, which demonstrated that coprecipitating contaminant proteins only constituted a very minor fraction of the column-eluate. Eluted CD9 antigen was therefore used to immunize rabbits. The high dose of the immunogen ensured the successful production of high titre polyclonal antibody, which later turned out to be essential for the isolation of CD9 positive clones from the λgt11 library. The specificity of the polyclonal antibody against

CD9 antigen was confirmed by Western blotting, by indirect immunoflurescence microscopy and FACS analysis, and most importantly, by peptide mapping of the protein immunoprecipitated with the antibody.

While CD9 antigen purification and animal immunization were carried out, a NALM-6 cDNA library was constructed in λgt11 expression vectors. A library with a 4 x 10^5 p.f.u. packaging titre was obtained from 10 μg of poly A^+ mRNA. At the time the library was constructed, λgt11 was just emerging as a promising expression vector for cDNA cloning with antibody probe. Experiments were conducted to compare the different antibody screening methods in order to find the best method suited for the study. As a result, gelatin was chosen as the blocking agent, and horseradish peroxidase conjugated goat anti-rabbit second antibody was chosen to visualize the first antibody. Since mAb 50H.19 was able to recognize the denatured CD9 antigen on nitrocellulose filters, it appeared to be a good candidate for use in λgt11 library screening. The library was initially screened with mAb 50H.19, which did not result isolation of any truly positive clones. A surprise came when it was found that although the monoclonal antibody was able to recognize the SDS- and heat-denatured CD9 antigen, it would fail to do so once the antigen was reduced. Polyclonal antibody was thus absolutely necessary for isolating the CD9 positive clone from λgt11 libraries.

Screening of the NALM-6 λgt11 library and of the HepG2 library resulted in the successful identification of a positive clone from the latter. The clone identification based on recognition of the CD9 epitope was confirmed by rescreening the positive clone with the polyclonal antibodies preadsorbed with various cell lines differing in CD9 expression, a novel approach not reported by other investigators. The progress of the project was impeded, however, by the inability to excise the 1.3 kb cDNA insert from the phage DNA for the purpose of subcloning, which turned out to be a very common, yet unpublished, problem encountered in cloning in λgt11 (personal communication),

the cause of which can still not be explained satisfactorily. The problem was fortuitously solved when the cDNA, together with some flanking phage DNA was subcloned into a plasmid vector, and as a control, the recombinant plasmid DNA digested with Eco RI. The 1.3 kb cDNA insert could then be cut out and subcloned into the M13 sequencing vector, although the recombinant M13 carrying the insert could not be deleted, probably due to the same factor that prevented the excision of the insert from the phage DNA in the first place. Sequence specific oligonucleotide primers were then used as primers for both strands of the entire insert.

Sequence analysis of the first cDNA revealed that it possessed an open reading frame that extended all the way to the 5' end , and the open reading frame has a potential to encode a 32 kDa protein. A potential poly A addition signal was located about 300 bp downstream from the translation stop codon. Eight potential translation start codons were present. Closer analysis of the start codons identified the fifth ATG as the possible start site, since it had a very good match with the consensus eukaryotic functional start codon, and neighboring sequence.

Search of the human gene data base demonstrated that the cDNA sequence is unique. Furthermore, the hydrophobicity plot indicated that protein product did not have a transmembrane domain, which is consistent with the nonintegral nature of CD9 protein. Most significantly, the sequence downstream from this particular start site encoded a protein which possessed a predicted proteolytic pattern that almost completely matched that obtained from peptide mapping data. These findings strongly suggest that the cloned cDNA is very likely to encode CD9. Preliminary characterization of the antibody directed against the synthetic peptide also seems to support this conclusion. The protein belongs to the immunoglobulin supergene family on the basis of significant homology of conserved residues which would also be consistent with a membrane-located protein involved in cell adhesion. Southern blotting using the cDNA as

hybridization probe indicated that the CD9 gene may exist as multiple copies in the genome, or that it may belong to a multigene family, although the possibility of the existence of a single but very large gene cannot be excluded. Interestingly, Northern blotting identified CD9 mRNAs in cells lacking cell surface antigen expression, suggesting that the expression of the CD9 protein may be controlled at both transcriptional and post-transcriptional levels, a concept also supported by Western blotting which detected a 22 kDa protein in the lysates of surface-CD9 negative cells. Northern blotting also indicated that CD9 mRNA is inducible upon T-cell stimulation and upon cell transformation suggesting that CD9 may indeed play a role in cell activation.

Various experiments were carried out to further confirm the authenticity of the CD9 cDNA clone isolated from the HepG2 λgt11 library with the polyclonal antibody. The result of the *in vivo* expression of CD9 cDNA through transfection was difficult to interpret because of the presence of background CD9 protein. Final authentication of the CD9 cDNA will therefore have to await the results from microsequencing the internal peptide generated from proteolytic digestion of highly purified CD9 antigen since the amino-terminus of the native protein is blocked, and from analyzing the specificity of the antibodies raised against peptides synthesized on the basis of hydrophilic regions of the predicted CD9 protein.

Finally, the CD9 cDNA was used to isolate a second clone from the NALM-6 λgt11 library. A similar problem was encountered in excising the insert from the phage DNA. Since subcloning the 0.7 kb insert plus the flanking phage DNA still did not solve the problem, attempts are being made to sequence the 0.7 kb insert by λgt11 sequencing primers using the double-stranded 2.8 kb fragment as templates. Alternatively, the 2.8 kb fragment would be subcloned directly into the M13 vector, and the sequencing carried out using the λgt11 sequencing primers as well.

In conclusion, the aim of this study was successfully achieved by purifying large amounts of CD9 antigen to raise polyclonal antibody, which enabled the final isolation of a CD9-encoding cDNA. The clone confirmation is based upon rescreening the positive clone with preadsorbed antibodies, and more importantly on the almost complete match of the size of the predicted peptide to that established with the native CD9 protein. Final confirmation awaits the results from the experiments outlined.

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